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CD45 in Lymphocyte Adhesion and T Cell Activation

by

©

Xiu-Yan Jiang

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science.

Department of Laboratory Medicine
and Pathology

Edmonton, Alberta

Fall, 1995

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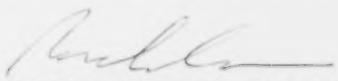

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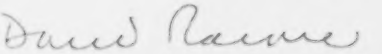

Dr. David Rayner

Date

sept 5 '95

DEDICATION

This thesis is dedicated to my parents and my husband who are always an inspiration to me, and whose encouragement and support were invaluable.

ABSTRACT

The CD45 cluster of antibodies recognizes a family of trans-membranous molecules expressed exclusively on nucleated hemopoietic cells. Unrestricted CD45 Mabs recognise all members of the family, while CD45R Mabs react with restricted subsets.

In this study, we employed a panel of CD45 and CD45R Mabs to investigate two aspects of CD45 function: lymphocyte aggregation and T cell activation. Our results show that some CD45 and CD45R Mabs are able to induce homotypic aggregation of a number of lymphoid cell lines. This is an active process and involves both LFA-1-dependent and LFA-1-independent pathways. Some of the aggregation can be inhibited by blockers of tyrosine phosphatase. The same aggregation that can be inhibited by such blockers is calcium-dependent as suggested by inhibition by EDTA. This study suggests that the CD45 molecule is an important player in cell-cell interaction, most likely through its activity as a tyrosine phosphatase.

We also show that some CD45 and CD45R mabs are capable of inhibition of OKT3-induced human peripheral T cell activation. The main target for inhibition appears to be the CD8+ cytotoxic T cell subset. A possible mechanism involves dephosphorylation of a 100-110 KDa tyrosine phosphorylated protein. This study suggests that CD45 may be an appropriate target for immuno-modulation and that certain CD45 Mabs may be useful in the prevention and reversal of allograft rejection.

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ABBREVIATIONS

ALCL	Anaplastic large cell lymphoma
AEC	3-Amino-9-Ethylcarbazole
CD	Cluster designation
Fig	Figure
g	Gram(s)
h	Hour(s)
IPB	Immunoprecipitation buffer
KDa	Kilodalton
Kb	Kilobase
LCA	Leucocyte common antigen
LFA-1	Leucocyte function-associated antigen-1
Mab(s)	Monoclonal antibody
MW	Molecular weight
MNC	Mononuclear cell(s)
MAP	Mitogen-activated protein
PBL	Peripheral blood lymphocyte
PBS	Phosphate-buffered saline
PTPs	Protein tyrosine phosphatases
PI3	Phosphoinositide 3
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
TBS	Tris buffered saline
TTBS	Tween 20 Tris buffered saline
Tris	Tris(hydroxymethyl)aminomethane

CHAPTER 1

INTRODUCTION

Background

Leucocyte common antigen (LCA, CD45) is a family of receptor-like transmembrane proteins expressed exclusively by cells of hematopoietic origin (1). The discovery made by Charbonneau et al (2) that the tandem repeats in the cytoplasmic domain of CD45 had significant sequence similarity with a placental protein tyrosine phosphatase (PTPase) has been instrumental in shaping our view of how this important family of regulatory enzymes functions. Recent work has confirmed that CD45 plays an important role in lymphocyte function. However, there remain significant questions regarding its precise function and further study of CD45 may increase our understanding of transmembrane PTPs.

Genomic Structure of CD45

The CD45 gene maps to chromosome 1q31-32 in human (3). The gene is composed of 33 exons encoding a protein of 1291 amino acids and is approximately 110 Kb in length (Fig.1)(4). Transcription appears to be initiated at either of two exons, 1a or 1b that encode most of the 5′ untranslated region and all of the leader sequence. The complete amino acid sequence of CD45 has been determined from the analysis of cDNA clones (5). All CD45 isoforms follow the same structural scheme: an extracellular domain of either 391, 438, 486, 504 or 552 amino acids (depending on the patterns of exon splicing used) that are encoded by exons 3 to 15, a transmembrane region of 22

amino acids that is encoded by exon 16, and a large cytoplasmic domain of 707 amino acids encoded by exons 17 to 32. Exon 33 is the largest exon and encodes the remaining protein of the cytoplasmic domain and a 3' untranslated region of 1.1 Kb. The CD45 family is generated by alternative splicing of exons 4, 5 and 6. The differential use of these three exons can potentially generate eight different mRNAs (Fig.2)(6-8).

Structure of the CD45 protein

The CD45 molecule can be considered in terms of three distinct segments: an external domain, a cytoplasmic domain, and a membrane-spanning structure (Fig.3). The external domain is divided in at least two subdomains: an amino-terminal region containing O-linked carbohydrate attachment sites and two cysteine-rich regions (1). The O-linked region, which is rich in small aliphatic amino acids and heavily glycosylated, spans the three so-called 'variable regions ' (commonly labelled A, B, and C) which are encoded by exon 4, 5, and 6 (Fig.2). The cysteine-rich regions are localised between the O-linked region and the membrane spanning region, and are approximately 100 and 220 amino acid long. Each contains eight cysteines which are also heavily glycosylated. A 20 amino acid loop structure links the two cysteine regions (1). The external domain is less conserved among mammals. This would indicate that it is not the precise protein structure that is

of importance. The membrane spanning region consists of 22 amino acids. The cytoplasmic domain consists of two subdomains that are composed of 300 amino acids each, and a unique C-terminal tail. In contrast to the external domain, the cytoplasmic domain is highly conserved (up to 90%) among mammals. The two subdomains contain significant homology to placental tyrosine phosphatase (2,9,10).

Ultrastructural studies have demonstrated that CD45 protein consists of a globular structure of 12nm, representing the cytoplasmic domain and a rod-like structure of 18nm, representing the external domain (11).

Using SDS-electrophoresis and immunoblotting, CD45 proteins can be separated into four distinct bands of 220, 205, 190, and 180 KDa. Streuli et al (12) showed that the molecular weight of CD45 can be predicted on the basis of the number of variable regions expressed. Therefore, the 220 KDa species corresponds to the CD45 isoforms expressing all three variable regions (ABC), and the 180 KDa species corresponds to the CD45 isoform expressing none of the variable regions (O). The 205 KDa species corresponds to the CD45 isoforms with two variable region expressed (AB, BC, or AC), and the 190 KDa species corresponds to the CD45 isoforms with one variable region expressed (B or C).

Function of CD45

Consistent with the presence of homology between the

cytoplasmic domain of CD45 and placental tyrosine phosphatases, CD45 protein has been shown to possess intrinsic protein tyrosine phosphatase (PTPase) activity (2,9,10). CD45 PTPase contains two domains, the membrane proximal D1 domain and the membrane distal D2 domain. Only the D1 domain has enzymatic activity. The D2 domain may play a role in regulating PTPase activity (13,14). The analysis of CD45-deficient cell lines and CD45 knock out mice has shown that CD45 functions to positively regulate lymphocyte signal transduction (15) and plays a role in early lymphocyte activation (16,17) and in normal T cell development (15,18-21). PTPases can counter the actions of protein tyrosine kinases (PTKase). PTPases, together with PTKases, play a critical role in protein tyrosine phosphorylation signal transduction pathways (22-24). Recent studies demonstrated that some PTKases, such as $p56^{lck}$ and $p59^{fyn}$ proteins, are the physiological substrates of CD45 in T cells (25-27). CD45 may exert its biological effect via dephosphorylating PTKases such as $p56^{lck}$, $p59^{fyn}$, or the CD3 zeta chain. Tyrosine phosphorylation has a fundamental role in regulating calcium levels (28). CD45 deficient T cells do not manifest an early increase in intracellular calcium after stimulation (29).

The external domain is suspected to function as a receptor for one or more as yet unknown ligands. However, the exact interactions have not yet been elucidated. The difference in structure among the external domains of the different CD45

proteins probably determines the specific target stimuli for the different cell types expressing CD45.

CD45 is also implicated in B cell activation (30, 31) but apparently not in B cell development. The B cell antigen receptor can transduce a signal only if cells express CD45.

CD45 molecules are also potent inhibitors of natural killer cell cytolysis (32-34). This may be mediated through carbohydrate structures on the external domain of CD45 proteins (35).

Characterization of CD45 Cluster Antibodies

CD45 is a family of major glycoproteins. There are at least four different isoforms with molecular weights of 180, 190, 205, and 220 KDa (36). Antibodies reactive with CD45 isoforms can be divided into two types. Those that recognize epitopes shared by all CD45 isoforms have been termed CD45, whereas antibodies that only react with epitopes encoded by the variable exons of CD45 are defined as CD45R (for restricted) (37). CD45R can be subdivided into CD45RA, CD45RB, and CD45RC antibodies that recognize variable regions A, B, C, respectively. CD45R reactive with an epitope that is only present when the exon A, B, and C-encoded regions are absent are termed CD45RO. Anti-CD45RA Mabs precipitate bands of 220 and 205 KDa in immunoprecipitation experiments. Anti-CD45RB and CD45RC Mabs precipitate bands of 220, 205, and 190 KDa. Anti-CD45RO Mabs precipitate only the 180 KDa band. To date,

antibodies reactive with CD45RA, CD45RB, CD45RO and more recently CD45RC have been described (38,39). Frequently, the epitopes recognized by CD45 and CD45R Mab are sensitive to neuraminidase, consistent with the hypothesis that these Mabs recognize epitopes that are associated with carbohydrates, most likely terminal sialic acids (40). A number of in vitro studies have shown a wide range of effects of anti-CD45 Mab on T and B cell activation, cytotoxicity and cell adhesion (41,42).

Regulation of CD45 PTPase Activity

An understanding of how the PTPase activity of CD45 is regulated is crucial to define its role in signal transduction. Mechanisms by which CD45 activity might be reversibly modified include ligand binding, phosphorylation or other covalent modification, molecular interactions with other cell surface molecules or cytoskeletal components that lead to a redistribution of CD45 in the cell membrane, or the action of a specific intracellular inhibitor of PTPase activity. Although CD22 was initially reported to be a specific ligand for the CD45RO on human T cells (43), there is no evidence that CD22 modulates the activity of CD45 (44-46). To date, other attempts to identify putative ligands for different isoforms of CD45 have been unsuccessful. CD45 activity might be regulated through interaction with a variety of leucocyte cell surface molecules or cytoplasmic proteins such as LFA-1,

CD2, CD4, CD8, CD26, $p56^{lck}$, $p59^{fyn}$, fodrin etc.(47-50). Such associations might alter CD45 PTPase activity and lead to selective dephosphorylation of specific substrates brought into close proximity to CD45. Recently, $p50^{csk}$-mediated phosphorylation of CD45 was shown to result in increased CD45 PTPase activity (24).

The Purpose of This Study

CD45-related signals have been implicated directly and indirectly in a variety of lymphocyte functions (51-53). In this study, two aspects of lymphocyte function will be investigated: i.e. lymphocyte adhesion and T cell activation.

Direct interaction between cells is a characteristic feature of the immune system. A useful model system in which to explore various aspects of leucocyte adhesion and signal transduction is the phenomenon of homotypic adhesion. Several surface molecules have been shown to trigger homotypic adhesion involving B cells and T cells; these include CD9, CD11a, CD14, CD19 etc.(54-56). Some studies have shown that the CD45 molecule is involved in homotypic adhesion (57,58), but little is known about how different anti CD45 and CD45R Mabs are involved in T or B lymphocyte adhesion and possible signal transduction pathways. In chapter 2 of this thesis, the following will be addressed:

1. Anti CD45 and CD45R Mabs will be used to determine which epitopes are involved in homotypic adhesion of B and T cell

lines of different maturation stages.

2. The cell lines will be exposed to different reagents and the protein tyrosine phosphorylation patterns will be examined to evaluate possible mechanisms involved in the adhesion and signal transduction pathways.

The identification of signal transducers as crucial effectors in lymphocyte function suggests that modalities that intercept the signalling pathways may be of potential value in the treatment of graft rejection. Because T cell mediated immunity is a crucial component of the rejection phenomenon, it is reasonable to aim therapy only against this arm of the immune system (59). It is clear that CD45 has a pivotal role in lymphocyte activation by its PTPase activity. Although in vivo administration of anti-CD45 Mabs have been shown to prevent renal allograft rejection in mice with remarkable effectiveness and specificity (60,61), little is known about what determines the effect of anti-CD45 Mabs. An in vitro model of T cell activation in human peripheral blood lymphocytes might shed some light on this problem. In chapter 3 of this thesis the following will be addressed:

1. Different CD45 and CD45R Mabs will be used to define which ones augment or inhibit CD3 induced activation of PBL in vitro.

2. Subsets of T cells will be analyzed to determine which one is most affected by the CD45 Mab reagents.

3. The time of the addition of the antibodies and the concentration of the reagents will be varied to determine optimal conditions for the test.

4. Tyrosine phosphorylation patterns will be studied to determine how CD45 might be involved in the signal transduction pathways.

CHAPTER 2

CD45 IN LYMPHOCYTE ADHESION

Introduction

Adhesion molecules play a crucial role in the immune system (62). Leucocyte-leucocyte adhesion is a necessary component of many immunologic interactions (63). One useful model system in which to explore various aspects of leucocyte adhesion and signal transduction is the phenomenon of homotypic adhesion, which was initially described in response to phorbol esters (64). Lymphocyte homotypic adhesion can also be induced by Mabs binding to numerous surface antigens (55,65-67). LFA-1/ICAM-1 interaction plays a major role in homotypic adhesion (68,69), but other adhesion molecules are also involved (55,56,70). Several studies have shown that the CD45 molecule is involved in homotypic adhesion (57,58,71). The cytoplasmic domain of CD45 has a PTPase activity and may play a role in the regulation of hemopoietic cell growth (2,9,72). Cross-linking of CD45 with Mabs can deliver signals that either enhance or inhibit T cell activation depending on the stimulating pathways and the target cells (29,73,74). The exact mechanism of signal transduction via CD45 is still unknown, but it has been shown that CD45 regulates $p59^{fyn}$ and $p56^{lck}$ tyrosine kinases through its PTPase activity (75).

In this study, the ability of different CD45 and CD45R Mabs to induce the homotypic adhesion process was examined.

Materials and Methods

Mabs

The following Mabs were prepared in our laboratory: 4D11, 4C9, 2G1, MB1, MT2, MT3, 6G3, and 6B6. UCHL-1 Mab was kindly provided by Dr.P. Beverley, University College Hospital Medical School, London, UK. Mabs 4D11, 4C9, and 2G1 recognize CD45; Mabs MB1 and MT2 recognize CD45RA; Mabs MT3, 6G3 and 6B6 recognize CD45RB; Mab UCHL1 recognizes CD45R0. Mab CLB-CD18 reacts with the ß chain of LFA-1 (76) and was purchased from Cedarlane Laboratories, Ontario, Canada. Biotin-conjugated 4G10, an anti-phosphotyrosine Mab was purchased from Upstate Biotechnology Inc. New York.

Cells

Human T cell lines Jurkat and Molt-4, B cell lines Raji, Daudi and Ramos, stem cell line KG1a and anaplastic large cell lymphoma cell line (ALCL) Karpas were originally obtained from the American Type Culture Collection. Pre-B lymphoblastoid cell lines Hoon and Nalm 6 were a kind gift of Dr. M. Letarte, Department of Immunology, University of Toronto. Hodgkin cell line L428 was a kind gift by Dr. V. Diehl, Department of Oncology, University of Cologne. B cell lines Rose, Schi, Zyl, VER and Hodgkin cell line DEV were newly established in our laboratory. All cell lines were maintained in RPMI 1640 supplemented with 10% heat-inactivated FCS, 2 mM glutamine,

and 50 um/ml penicillin and 50 ug/ml streptomycin.

Reagents

Verapamil and sodium orthovanadate were purchased from Sigma.

Adhesion Assay

Adhesion assays were performed in flat-bottomed culture plates with 96 wells (Falcon). Mabs to be tested were added to 2-3 x 10^6 cells in 150 ul of assay medium and cultured at 37°C in a 5% CO_2 humidified atmosphere. All were assayed in triplicate and each experiment was performed at least twice. Each well was evaluated every hour up to 24 hs and read independently by two observers.

A semi-quantitative scoring method of adhesion adapted from Rothlein and Springer was used (64): 0 for no adhesion or cell cluster formation; 1+ indicating that less than 10% of the cells were in aggregates; 2+ indicating that < 50% of the cells were in aggregates; 3+, up to 90% of the cells were in small, loose clusters; and 4+, 90% of the cells were aggregated in large clusters. Photographs were taken with a Leitz Wetzlar camera. For temperature experiments, plates were placed at 37°C, room temperature, and 4°C, respectively. For inhibition assays, cells were pretreated with sodium orthovanadate (20-40 umol/L) or verapamil (10 umol/ml) for 2 hs at 37°C before the addition of the adhesion inducing Mabs.

Immunocytochemistry Staining

The various cell lines were tested for LFA-1 expression with the CLB-CD18 Mab on cytospin slides and positively staining cell lines were pretreated with this Mab for 1 h at 37°C before adding the different CD45 Mabs. Peroxidase staining was obtained with a mixture of hydrogen peroxide and AEC for 13 min.

Detection of Tyrosine Phosphorylated Proteins

Cell lines with or without sodium orthovanadate were incubated with the indicated Mabs for up to 24 hs. The cells were then washed twice with ice-cold PBS and lysed by adding IPB containing 10 uM Triethanolamin pH 7.8, 150 mM Nacl. 1% Nonidet P-40, 1 mM PMSF, and 20 ug/ml tyrosine inhibitor for 45 min. Acrylamide concentration of the separation gels was 10%, and the proteins were electrophoretically transferred from the gel onto nitrocellulose at 100 V for 1 h. The filters were blocked by a solution of 3% gelatin in TBS for 1 h and then washed twice in TTBS and incubated with the anti-phosphotyrosine Mab 4G10 (1.0 ug/ml) in antibody solution (1% gelatin in TTBS) for 1 h at room temperature. After the filters were washed twice in TTBS, they were incubated with horseradish peroxidase-conjugated avidin (Bio-Rad, Richmond, CA) diluted 1:1000 in antibody solution for 1 h at room temperature, washed twice in TTBS, and then placed in a buffer containing 4 mg/ml 3-amino-9 ethylcarbazole and 0.005% H_2O_2

for 5 to 10 min. Enzymatic color development was stopped by rinsing the filters in distilled water.

Results

Homotypic Cell Aggregation is Induced by Some CD45 and CD45R Mabs on Some Cell Lines

To identify the CD45 Mabs which could induce lymphocyte homotypic cell aggregation, a panel of CD45 Mabs were screened for their ability to induce cell aggregation. Table 1 shows that some Mabs were potent inducers of homotypic cell aggregation in a variety of cell lines with different degrees of aggregation. However, pre-B cell line Nalm 6, B cell lines Daudi, VER, Zyl, Raji, and Schi, ALCL cell line Karpas, and Hodgkin's cell lines L428 and DEV did not show induction of homotypic cell aggregation by any of the CD45 and CD45R Mabs tested.

Small amounts of Mab at subsaturating doses (1 ug/ml) were potent activators of aggregation and increasing the amount of Mab did not result in further significant increases in aggregation. The rate of aggregate formation was variable between cell lines and Mabs (Fig. 4-8). Most aggregate formation was observed as early as 1 to 2 hs, except for the cell line Molt 4 responses to 6B6 Mab that required more than 6 hs till the start of aggregation (data not shown). When cultured with the different Mabs, cells gradually aggregated

into large spheroids, often reaching 200 to 300 um in diameter, and in some cases up to 500 um (Fig. 9). Based on the time course and direct observation under the microscope it can be excluded that the spheroids were simply the progeny of single cells that failed to separate postmitotically. When pipetted, the cells remained in large tightly adherent clumps.

CD45 Induced Aggregation Requires a Physiologic Temperature and Ca^{2+} Cations.

We next studied the effect of temperature on aggregation induced by CD45 Mabs. For this purpose, the cell lines were incubated with CD45 Mabs at 37°C, room temperature, and 4°C, respectively. We found that no aggregation could be detected at 4°C and that aggregation was reduced to 50% of the value obtained at 37°C if the assay was conducted at room temperature. No adhesion of the Molt 4, Hoon, and Ramos cell lines was present at room temperature, indicating that the homotypic adhesion requires energy. The results indicate that CD45-mediated homotypic aggregation is a specific, energy dependent mechanism and is not the result of passive cell aggregation or of antibody crosslinking of the cells.

Lymphocyte aggregation induced by Mabs has been reported to be dependent on Ca^{2+} cations (56,64). We therefore employed verapamil, a calcium channel blocker or Ca^{2+} antagonist, to investigate the Ca^{2+} requirements of the various cell lines and found that some, but not all aggregations were inhibited

by the presence of verapamil (Table 2). The concentration of verapamil was not a limiting factor because increasing the concentration of verapamil from 10 umol/ml to 40 umol/ml did not further inhibit aggregation (data not shown). The results show that CD45 induced aggregation of some but not all cell lines requires Ca^{2+} cations and that calcium channel blocker verapamil can abrogate CD45 Mab-mediated aggregation of the Ca^{2+} dependent lines.

LFA-1/ICAM-1 plays a role in some CD45 induced homotypic adhesion

The CD11a/CD18 adhesion system has been suggested to be important in a wide range of T and B cell functions (77,78). Many surface molecules known to induce homotypic aggregation are known to activate the LFA-1 adhesion pathway (79,80). We therefore investigated the role played by LFA-1 in CD45 Mab-mediated aggregation. We first stained Jurkat, Molt-4, Hoon, Ramos, Rose and KG1a cells with an antibody (CLB-CD18) to the ß chain of LFA-1 since Mab to the ß-chain of LFA-1 is known to be more effective than Mab to the α-chain in homotypic adhesion (65). We found that only Molt-4 and KG1a cells expressed LFA-1 (data not shown). Furthermore, we pretreated Molt-4 and KG1a cell lines with CD18 Mab (25ug/ml) followed by incubation with the different CD45 Mabs. Table 3 demonstrates that 6B6-induced Molt-4 cell aggregation and MB1, MT2, and 4C9 induced KG1a cell aggregation were totally

abrogated, indicating that those Mabs may induce aggregation through the LFA-1 adhesion pathway.

Vanadate prevents some CD45 Mab-mediated aggregation

Previous studies have shown that CD45 has tyrosine phosphatase activity (2,9,72). To address the potential role of tyrosine phosphatase in CD45 Mab-mediated aggregation we used the phosphatase blocker sodium orthovanadate (20 uMol/ml) which has been widely employed to investigate the role of protein tyrosine phosphatases in signal transduction. The results (81-83) are summarized in Table 4. Some, but not all, CD45 Mab-mediated aggregation was completely blocked by vanadate. This indicates that the CD45 induced aggregation of some cell lines involves phosphatase-initiated signalling pathways. The presence of cell lines with vanadate resistant CD45 Mab-mediated aggregation suggests the existence of other signalling pathways which are independent of phosphatase activity.

Binding of some CD45 Mabs to lymphocytes results in changes in cellular protein tyrosine phosphorylation.

To further investigate the effect of the CD45 tyrosine phosphatase activity, total cellular tyrosine phosphorylated protein patterns were examined. Cell lines were incubated in media alone, media containing different CD45 Mabs, media containing Mabs that did not induce homotypic adhesion, and

media containing CD45 Mabs plus vanadate which inhibited CD45 Mab-mediated aggregation. Tyrosine phosphorylated proteins were detected by western blotting with a Mab specific for phosphorylated tyrosine (4G10). A "normal pattern" of tyrosine phosphorylated proteins was defined in unstimulated cells that were used as a control. As shown in Fig 10-14, many proteins were spontaneously phosphorylated on tyrosine residues in the unstimulated cells. Treatment of Molt 4 cell line with 6B6 Mab for up to 24 hs altered the patterns, most notably there was increased phosphorylation of bands at 16 and 18 kDa. Incubation with 6B6 plus vanadate resulted in the absence of these bands, indicating that phosphorylation of those proteins might be involved in cell aggregation. Fig 11 illustrates that the pattern of phosphorylation of the Hoon cell line after incubation with 4D11 and 6G3 differed significantly from the pattern observed after incubating the cells in media alone. Increased phosphorylation of a band at 42.7 kDa after incubation with 4D11 can be observed, suggesting that phosphorylation of this protein may be important for cell adhesion of Hoon. Decreased phosphorylation of a band at 67 KDa was found after pretreatment of Hoon cells with 4D11 plus vanadate, indicating that vanadate possibly inhibited Hoon cell aggregation by dephosphorylation of this protein. On the other hand, treatment of Hoon cells with 6G3 resulted in decreased phosphorylation of the band at 42.7 KDa and increased a band at 97.4 KDa. Treatment of the Ramos cell line

with 6B6 or 4D11 did not change the tyrosine phosphorylation patterns, suggesting that perhaps other adhesion mechanisms are involved (data not shown). Fig 12 shows that treatment of the Jurkat cell line with different Mabs resulted in distinct patterns of phosphorylation. Incubation with MT3 lead to increased phosphorylation of a 220 kDa protein and pretreatment of the cells with vanadate resulted in decreased phosphorylation of the 220 KDa and 97.4 KDa proteins. 4C9 and 6G3 Mabs induced phosphorylation of an 85 KDa protein that was not present after coincubation with vanadate. 4D11, 6B6, and 2G1 did not alter any patterns. Therefore, different Mabs acting on the same cells lead to phosphorylation of different proteins through distinct mechanisms. This phenomenon also occurred when KG1a and Rose were incubated with anti CD45 reagents. Fig 13 demonstrates that treatment of the KG1a cell line with 4C9 and MT2 induced phosphorylation of an 85 KDa protein. In addition to this 85 KDa protein, 6G3 also induced phosphorylation of the 97.4 KDa protein. This 97.4 KDa band also appeared after pretreatment of KG1a cell with vanadate followed by MB1 Mab. The 6B6 Mab did not induce tyrosine phosphorylation of KG1a. As shown in Fig 14, a 110 KDa protein was phosphorylated after treatment of the Rose cell line with 6G3. Pretreatment of Rose cells with vanadate followed by 6G3 decreased this band but increased the intensity of 200 KDa and 116 KDa bands. UCHL-1, 2G1, and MT3 did not alter the patterns compared with control Rose cells.

In summary, the data suggest that CD45 Mabs can induce cell adhesion through different pathways as reflected by alterations in tyrosine phosphorylation patterns of a number of proteins. In addition, there are pathways that appear not to involve detectable changes in tyrosine phosphorylation.

CHAPTER 3

CD45 IN T CELL ACTIVATION

Introduction

The T cell antigen receptor complex (TCR) mediates signals that can result in activation, anergy, or apoptosis; the outcome depending on the T cell differentiation state and the nature of signals transmitted by co-receptors (84-86). The TCR comprises a clonotypic α-ß or *y*-*δ* heterodimer, associated non-covalently with the invariant *y*, *δ* and *ε* polypeptides of the CD3 antigen, and with two additional polypeptides, zeta and eta (87-89). The polypeptides that associate with the α-ß heterodimer are involved in mediating T cell responses upon antigen recognition (90-92). The biochemical consequences of antigen binding to the α-ß heterodimer can be mimicked using mitogenic monoclonal antibodies that recognize epitopes on the CD3 antigen (93,94). Stimulation of the TCR complex induces the rapid phosphorylation of tyrosine residues in several proteins (95,96). Genetic experiments have established that the induction of protein tyrosine kinase (PTKase) activity is essential for the signal transduction function of the TCR (97,98). However, phosphorylation on tyrosine residues is also determined by tyrosine phosphatases (17,29,30). The best characterized lymphocyte tyrosine phosphatase is CD45. The CD45 molecule is a transmembrane glycoprotein expressed on the surface of all haematopoietic cells and their precursors except mature erythrocytes, plasma cells and megakaryocytes (1,2). The intracellular region of CD45 is composed of two tandemly repeated PTPase domains (2). The recognition of the

CD45 molecule as a tyrosine phosphatase has intensified interest in the role of tyrosine phosphorylation in lymphocyte activation (2,9). Haematopoietic cell lines deficient for CD45 expression are defective in antigen receptor-mediated activation (16,17,30), suggesting that dephosphorylation of other signal molecules by CD45 is crucial for signal transduction.

The major problem in organ transplantation is rejection of the transplanted organ (99,100). Several patterns of rejection can occur in solid organ transplants (101), but one of the most important aspects is T cell mediated immunity (102). Therefore, immunosuppressive agents that result in reduced T cell function and numbers are routinely used to minimize rejection. Such agents include cytotoxic drugs with specificity for T cells, immunosuppressive drugs, and antibodies directed against T cell surface molecules (103,104). Since there is an abundance of CD45 on the surface of lymphocytes and since its role in lymphocyte activation is both early and essential, it is a reasonable hypothesis to assume that Mabs against CD45 might be of value in the suppression of rejection. In fact, several previous studies have shown that manipulation of CD45 by Mabs can perturb T cell function (105). Anti-CD45 Mab used alone has also been shown to inhibit CD3-mediated T cell activation (106,107).

In this study, we demonstrate that anti-CD45RB Mabs (6B6, 6G3, MT3), anti-CD45RO Mabs (UCHL-1, A-6, and OPD4), and CD45

Mabs (4D11, 4C9, and 2G1) inhibit OKT3-mediated peripheral blood T cell activation in vitro, whereas another CD45RB reagent (PD7/26) and one CD45RA Mab (MT2) have no effect on T cell activation and CD45RA Mab MB1 and CD45 reagent 4F9 have co-stimulatory effects on OKT3-induced activation of T cells.

Analysis of subsets of T cells showed that CD8+ T cells are most affected by CD45 Mabs. It is well known that the CD8+ T cells are crucial and directly involved in the lysis of graft tissues (102). In addition, we showed that a 100-110 KDa tyrosine phosphorylated protein is important in the inhibition of OKT3-induced T cell activation by some CD45 Mabs. The data suggest that CD45RB and CD45RO may be appropriate targets for in vivo immunotherapy and may also provide some clues to study how CD45 Mabs affect signal transduction pathways.

Materials and Methods

Cells

Human mononuclear cells (MNC)(≈ 80% T lymphocytes) were isolated from heparin-treated blood of healthy donors by Ficoll-Hypaque (Pharmacia) density gradient centrifugation. Cells were maintained in RPMI1640 medium containing 10% FCS, penicillin (100 U/ml), and streptomycin (50 U/ml) for use in analytical experiments.

Monoclonal Antibodies and Fluorochromes Used

The OKT3 cell line was obtained from the American Type Culture Collection (Rockville, MD). This antibody was used for cell stimulations (107). Anti-CD45RO Mab UCHL-1 was kindly supplied by Dr. Peter Beverley (University College, London, UK). Anti-CD45RO Mab OPD4 (108) was purchased from DAKO, Denmark. Anti-CD45RO Mab A-6 (109) was purchased from Zymed Laboratories Inc, CA. Anti-CD45RB Mabs 6B6, 6G3, and MT3 were produced in our laboratory. Anti-CD45RB Mab PD7/26 was kindly provided by Dr. David Mason, Oxford, U.K. Anti-CD45 Mab 4F9 was kindly provided by Drs Takahashi and Kikuchi, Sapporo, Japan. Anti-CD45 Mabs 4D11, 4C9, and 2G1 were produced in our laboratory.

The following antibodies were used for staining of cell surface molecules: PerCP-anti-CD4, PerCP-anti-CD8, FITC-anti-CD25, and PE-anti-CD69 were purchased from Becton Dickinson. Anti-CD69 and anti-CD25 (IL-2 receptor) were used as indicators of early and late T cell activation respectively (110,111). Biotin-conjugated anti-phosphotyrosine Mab 4G10 (112) was purchased from Upstate Biotechnology Inc.

Functional Studies

Assessment of the effect of CD45 and CD45R reagents on OKT3-induced T cell activation.

Mononuclear cells (MNC) were suspended at a concentration of 10^6/ml and then placed in 5 ml round-bottomed tubes. The MNC

were cultured with different CD45 and CD45R Mabs in the presence or absence of OKT3 for 1, 2, 3, and 4 days, which allowed the kinetics of the experiments to be assessed. All cultures were incubated at 37°C in a 5% CO_2 humidified atmosphere.

To determine the appropriate concentration of CD45 Mabs to exert their function, different amount of Mabs were added to MNC.

Flow Cytometry

Flow cytometric analysis was performed with a FACScan (Becton Dickinson). Approximated 10^6 cells/ml were washed with ice-cold PBS and then stained with the antibodies on ice for 30 min. After washing with ice-cold PBS, cells were fixed using 1% paraformaldehyde and stored at 4°C until analysis. Viable lymphocytes were gated according to their forward and side-scatter properties. Data from 10,000 cells per test were analyzed with LYSYS II software (Becton-Dickinson). The percentages of positively stained cells were determined taking into account the results of control experiments.

To compare the effects of CD45 Mabs on OKT3 induced T cell activation, the percent inhibition or stimulation in all experiments was calculated as follows:

$$\% \text{ inhibition (or stimulation)} = \frac{I}{S} \times 100$$

I= the percentage of positive cells induced by different CD45 and CD45R Mab in the presence of OKT3 Mab (CD3), and S= the percentage of positively stained cells induced by OKT3 Mab alone.

Phosphotyrosine Western Blotting

Immunoblotting was performed, as previously described, with minor modifications (113). Briefly, cells were treated, as above, with Mabs for different time intervals (15 min to 24 hs). MNC were washed three times with ice-cold PBS and lysed by adding IPB containing 10mM Triethanolamine pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 1mM PMSF, and 20ug/ml tyrosine inhibitor for 45 min. After lysis at 4°C, the nuclei were pelleted and the supernatants subjected to SDS-PAGE on a 10% gel as previously described (95,114,115). Proteins were transferred to 0.45 uM nitrocellulose and the filters were incubated in TBS containing 3% gelatin for 1 h or overnight. The filters were then washed three times in TTBS and incubated with an anti-phosphotyrosine Mab (4G10, 1.0 ug/ml in TTBS containing 1% gelatin) for 1 h at room temperature. To detect Mab binding, the blots were washed three times with TTBS and incubated for 1 h with biotinylated anti-mouse immunoglobulins (BioGenex, CA) diluted 1/100 in TTBS containing 1% gelatin, washed three times in TTBS, and then incubated for 1 h with streptavidin peroxidase (BioGenex, CA) diluted 1/100 in TTBS containing 1% gelatin, washed three times in TTBS, and then

placed in a mixture of hydrogen peroxide and 3-amino-9-ethylcarbazole (AEC) (Sigma, St, Louis,USA). Enzymatic color development was stopped by rinsing the filters in distilled water.

Immunohistochemistry

To determine at which point in time MNC were positively stained with biotin-conjugated 4G10, immunohistochemistry was performed. MNC treated with OKT3 alone were spun down. The slides were fixed in acetone for 10 min. 4G10 (10 ug/ml) in PBS was then applied to the fixed cells and incubated for 1 h at room temperature. After three washings with PBS, the cells were treated with streptavidin peroxidase diluted 1/15 for 30 min (BioGenex, CA). Peroxidase staining was obtained with a mixture of hydrogen peroxide and AEC for 13 min.

Results

Inhibition of OKT3-mediated T cell activation by CD45RB (6B6, 6G3, MT3), CD45RO (UCHL-1, OPD4,and A-6), and CD45 (4D11, 4C9, and 2G1).

Lower case t test was performed comparing different CD45 and CD45R Mabs plus OKT3 with OKT3 stimulation alone and an ANOVA with Wilcoxon signed rank test was also performed to compare different effects among CD45 and CD45R Mabs in OKT3-induced T cell activation. None of the CD45 and CD45R Mabs alone lead to T cell activation (Fig.15). CD45RB (6B6, 6G3,

MT3), CD45RO (UCHL-1, OPD4, and A-6), and CD45 (4D11, 4C9, and 2G1) all block OKT3-induced T cell activation on day 4 in all experiments at a dose of 2-5 ug/ml. Among them UCHL-1 and 6G3 were the strongest inhibitors (Fig. 16-18). Furthermore, 6G3 plus UCHL-1 were able to synergically inhibit T cell activation, and a combination of 4D11, 6G3 and UCHL-1 was able to completely inhibit OKT3-induced T cell activation to a level comparable to the control without OKT3 Mab stimulation (Fig. 19). However, another CD45RB Mab (PD7/26) and a CD45RA Mab (MT2) Mab had no effect on the OKT3 induced T cell activation (Fig. 16,20). MB1 (CD45RA) and 4F9 (CD45) Mabs had co-stimulatory effects on OKT3-induced T cell activation (Fig. 18, 20).

CD45RB 6G3 Mab inhibited OKT3-induced T cell activation in a dose-dependent fashion up to a concentration 2-5 ug/ml with no further significant increase in inhibition at 10-25ug/ml (Fig. 21).

The kinetics experiments showed that UCHL-1 (CD45R0) consistently blocked OKT3 induced T cell activation from day 1 to day 4, whereas 6B6 and 6G3 Mabs (CD45RB) began to inhibit T cell activation from day 2 and significantly blocked T cell activation after day 3. 6G3 plus UCHL-1 and 6G3, plus 4D11 and UCHL-1 blocked T cell activation from day 1 and the latter combination completely inhibited T cell activation from day 1 as measured by CD25 expression. In contrast, CD45 4F9 Mab co-stimulated OKT3-induced T cell activation from day 2 (Fig 22).

All Mabs shown significantly inhibited T cell activation on CD69 expression from day 2 except that 6G3, 4D11 plus UCHL-1 exerted its blocking effect from day 1 (Fig 23).

CD45 Mab Mainly Inhibits CD8+ T Cell Subset Activation

Since 6B6, 6G3, MT3, 4D11, 4C9, 2G1, UCHL-1, OPD4, A-6, MB1, and 4F9 were capable of effects on OKT3-induced T cell activation, we tried to determine whether a particular T cell subset was being inhibited or stimulated. An ANOVA with Wilcoxon signed rank test was performed, and as shown in Figure 24-37, CD8+ subset of T cells was mainly inhibited. 6B6, 6G3, MT3, UCHL-1, A-6, 4C9, 2G1, 6G3 plus UCHL-1, and 4D11 plus UCHL-1, mainly inhibited CD25 expression on CD8+ T cells. UCHL-1, OPD4 and 4C9 inhibited CD69 expression on CD4+ T cells. OPD4, 4D11, and 6G3 plus 4D11 and UCHL-1 inhibited CD25 expression on CD4+ and CD8+ T cells. 6B6, 6G3, MT3, A-6, 4D11, 2G1, 6G3 plus UCHL-1, 4D11 plus UCHL-1, and 6G3 plus 4D11 and UCHL-1 inhibited CD69 expression on both CD4+ and CD8+ T cells. 4F9 and MB1 had costimulatory effects on both CD4+ and CD8+ T cells.

A 100-110 KDa Tyrosine Phosphorylated Protein is Important in Inhibition of T Cell Activation

The CD45 molecule has been demonstrated to have PTPase activity (2,9,72). Dephosphorylation of critical tyrosine residues by CD45 may be important in signal transduction

pathways. To determine the role of CD45 Mabs in T cell activation, we stimulated MNC with OKT3 alone or with the different CD45 Mabs and control for 1, 2, 5, 10, 15, 20, 30 min and 24 hs. As shown in Fig. 38 and 39, the 100-110 KDa protein was constitutively phosphorylated in MNC stimulated with OKT3 after 10 min. This result was similar to previously published observations (93). However, this band was absent in MNC stimulated with OKT3 plus the different CD45 Mabs that also resulted in inhibition of CD25 expression (Fig.38 and 39). This suggests that this protein plays a role in the inhibition of OKT3-induced T cell activation by the CD45 and CD45R Mabs.

In order to determine at which point in time tyrosine phosphorylated proteins in MNC stimulated with OKT3 can be detected in cytospin preparation, immunohistochemistry was performed at 1, 2, 5, 10, 15, 30, 60 min. and then every two hours up to 24hs. The result showed that tyrosine phosphorylated proteins can be detected after 2 hs and have strong staining after 22 hs (data not shown). Therefore, compared with the western blotting, immunohistochemistry appeared less sensitive. Futhermore, pretreatment of MNC with vanadate 1 min followed by OKT3, tyrosine phosphorylated proteins can be detected after 1 min (Fig.40). This indicated that vanadate could inhibit PTPase activity leading to hyperphosphorylation of tyrosine proteins.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

Discussion

The functional role of the most abundant leucocyte surface molecule, the leucocyte common antigen (CD45), is not completely clear. In this study, we investigated the role of CD45 in two aspects of lymphocyte function, i.e activation and adhesion. In lymphocyte aggregation, both LFA-1/CD18-dependent and -independent mechanisms of adhesion could be activated by CD45 and CD45R Mabs. This supports previous observations (62) that suggest the CD45 molecule is involved in adhesion.

Involvement of different adhesion mechanisms in aggregation induced by CD45 and CD45R Mabs could be related to the engagement of different CD45 and CD45R epitopes and potential interactions with other cell surface molecules. This in turn may lead to the activation of different intracellular signalling pathways. In fact, at least two signal transduction processes have been identified in cell-cell adhesion. One involves protein kinase C which triggers LFA-1 adhesion, the other is a tyrosine kinase-dependent pathway which activates LFA-1 and other adhesion receptors (57). The adhesion-inducing Mabs can cause a conformational change leading to activation of the LFA-1 adhesion molecule (116).

The identity of the molecules that mediate the LFA-1-independent pathway of homotypic adhesion is presently unknown. Our data show that vanadate can inhibit some but not all CD45 Mab-induced aggregation. In addition, binding of some

CD45 Mabs to lymphocytes induced changes in the patterns of cellular protein tyrosine phosphorylation, with increased phosphorylation of some proteins and decreased phosphorylation of others (Fig.10-14). This suggests that tyrosine phosphorylation is involved in this process and that Mabs binding to CD45 may lead to activation of the cytoplasmic PTPase domain of CD45. The PTPase dephosphorylates substrates including PTKases that get activated and further phosphorylate other substrates resulting in upregulaton of a variety of adhesion molecules promoting homotypic adhesion. In most cell lines that were pretreated with vanadate followed by different CD45 Mabs some tyrosine phosphorylated bands disappeared. This suggests that vanadate inhibition of PTPase reduces PTKase activity by allowing unopposed phosphorylation of PTKase tyrosine residues resulting in decreased PTKase activity, or by some more complex intermediary set of interactions.

Interestingly, MT3 induced tyrosine phosphorylation of a 220 KDa protein which became much less prominent after pretreatment with vanadate in Jurkat cell line. Pretreatment of the Rose cell line with vanadate followed by 6G3 showed increased tyrosine phosphorylation of a 200 KDa protein (fig 14). Since 220 and 200 KDa proteins correspond to the MW of CD45 (2), it is likely that in these instances the CD45 molecule itself is phosphorylated by tyrosine kinases. It has previously been reported that $p50^{csk}$ is capable of phosphorylating CD45 on tyrosine (140-142).

Our data clearly demonstrate the complexity: binding of the same Mabs to different cell lines or different Mabs to the same cell line results in different tyrosine phosphorylation patterns. It can be hypothesized that each cell line has different signal transduction pathways after Mab binding to CD45 even with the same Mab. Because the specific kinase or target substrates for the tyrosine kinase dependent adhesion pathways in human lymphocytes have not been identified, it is difficult to directly assess the significance of dephosphorylation or increased phosphorylation of specific proteins. However, the results support the notion that CD45 has a crucial role in the regulation of cell-cell aggregation.

Lymphocyte aggregation induced by Mabs has been reported to be dependent on Ca^{2+} cations (56,64). Little is known about the relation between Ca^{2+} requirement and tyrosine phosphorylation in homotypic adhesion induced by CD45 Mabs. Our study showed that most aggregation induced by CD45 and CD45R Mabs through tyrosine phosphatase is calcium-dependent (Table 2,3). The findings suggest that activation of PTPase requires an increase in intracellular Ca^{2+}. It has also been reported that some aggregation induced by Mabs is Mg^{2+} dependent (65,117). Our data showed indeed that some aggregation induced by CD45 Mabs did *not* require Ca^{2+}, suggesting that in those instances aggregation may require Mg^{2+}.

The nature of the 220, 200, 97.4, 116, 110, 85, 67, 42.7, 18, and 16KDa proteins that became phosphorylated upon engagement of CD45 and CD45R or dephosphorylated upon pretreatment with vanadate remains to be determined. Possible candidates of similar molecular masses would include proteins known to be phosphorylated after activation through the CD3/TCR complex such as different members of MAP kinase family (about 40 KDa)(118), an 18KDa band corresponding to the zeta chain of CD3 (95), 220 and 200 KDa bands corresponding to the CD45 itself (120), a 97.4 KDa band, consistent with the MW of CD18 (LFA-1 ß chain) which was phosphorylated after leucocyte activation (121,122), an 85 KDa band corresponding to the PI3 kinase (123), a 67 KDa band corresponding to CD5 (124) and 116-, 110-, and 16- KDa proteins of unknown identity. Our data suggest that induction of tyrosine kinase activity is important in the signal transduction pathways in lymphocyte homotypic adhesion and one or more of the substrate proteins may serve as intermediate messengers in linking surface receptors and lymphocyte activation leading to homotypic adhesion. It would be interesting to investigate the putative tyrosine kinases involved in those signalling pathways.

In addition to its direct role as a signal transducer, CD45 plays a critical role in T cell activation via the TCR/CD3 complex. Our data show that CD45 and CD45R Mabs (6B6, 6G3, MT3, UCHL-1, OPD4, A-6, 4D11, 4C9, and 2G1) were able to inhibit OKT3-induced T cell activation. Among them, UCHL-1 was

found to result in the strongest inhibition of T cell activation, but CD45RB reagents 6G3 and 6B6 also had significant inhibitory activity. It has been reported (125) that in patients after kidney, heart, and liver transplantation, a clear-cut increase in the frequency of CD45RO+ T cells was detected during most episodes of acute graft rejection. Other studies (126,127) also have shown that following in vitro activation by CD3 Mabs, naive T cells stop expressing CD45RA and start to display the CD45RO isoform within 4 to 6 days. We found that the main target for inhibition by the CD45(R) Mabs is the CD8+ T cell subset. This is of importance in transplantation since the CD8+ T cells are believed to be the main players in the rejection event (102). Almost all CD8+ lymphocytes in the peripheral blood of kidney transplant patients were found to be activated, including 68% CD45RO+ cells with cytotoxic activity (128).

Our data also show that UCHL-1 exerted its blocking function on day 1 after OKT3 stimulation, whereas 6G3 and 6B6 had their major effect after 3-4 days. This suggests the involvement of different mechanisms and the possibility that UCHL1 and the CD45RB reagents might have a complementary effect. Indeed this was found and, moreover, combination with a third reagent from the CD45 common group led to almost complete inhibition of CD25 expression. Therefore, treatment with a cocktail of CD45(R) reagents carries great promise as an immunologic approach towards inhibition of graft rejection.

The mechanism whereby CD45 and CD45R Mabs exert their effects is still unclear. However, our experiments showed that a 100-110 KDa protein is important in inhibiting T cell activation. Phosphorylation of a 100-110 KDa protein after stimulation with anti-CD3 reagents has previously been shown (115). The 100-110 KDa protein was not phosphorylated after the addition of those CD45(R) Mabs (6B6, 6G3, UCHL-1)(Fig. 44,45) that also had the most prominent inhibitory effect on CD25. This indicates that the various Mabs inhibiting T cell activation may use the same signal transduction pathways in different T cell subsets. CD45RB Mab MT3 only mildly decreased the intensity of the 110-100 KDa band. MT3 showed significantly less inhibition of OKT3-induced T cell activation than 6G3 and 6B6. This indicates that the degree of inhibition may be associated with the degree of dephosphorylation of the 110-100 KDa protein. CD45 antibody 4F9 did not change the patterns of tyrosine phosphorylated proteins.

There are three possible mechanisms by which Mabs might inhibit T cell activation: First, Mabs may block or sterically hinder a "receptor" from binding a target cell ligand for CD45. This would prevent the activation of cytoplasmic domain PTPase CD45 which is necessary for activation of PTKases via dephosphorylation of tyrosine protein kinases, such as $p56^{lck}$ and $p59^{fyn}$ (75,129,130). Second, vital molecules may be "modulated" from the cell surface. Third, the Mabs may deliver

a negative signal to inhibit T cell function. The failure of the 110-100 KDa protein tyrosine phosphorylation might reflect failure of kinase activation. Alternatively, this protein might be rapidly dephosphorylated by an activated CD45. A limitation of the current study is that with immunoblotting it is not possible to determine whether the Mabs have activated or inhibited CD45 phosphatase activity. Ultimately answers to these mechanistic questions require isolation of the substrates and phosphate turnover studies. Data from many laboratories indicate that at least two Src family kinases, $p56^{lck}$ and $p59^{fyn}$, play active roles in TCR-CD3-induced signal transduction. $P56^{lck}$ is non-covalently associated with the cytoplasmic tails of CD4 or CD8 (131,132), while $P56^{fyn}$ is non-covalently associated with TCR (133). They have distinct functions in T-cell physiology but both are utilized for TCR-CD3 signal transduction (134-137). The catalytic activity and function of $p56^{lck}$ and $p59^{fyn}$ are under stringent control by a number of enzymes, including CD45 PTPase (25,75,129,138,139) and $P50^{csk}$ PTKase (140-142). Both of these act on a conserved C-terminal tyrosine residue [Tyr-505 in $p56^{lck}$ and Tyr-528 in $p59^{fyn}$]. When phosphorylated, these tyrosine residues are believed to bind to the src homology 2 (SH2) domain within the same molecule, thereby forcing the kinases into an inactive conformation (143-146). Autero et al (24) proposed a model for the interactions between CD45, $p50^{csk}$, and $P56^{lck}$ ($p69^{fyn}$) (Fig.41 and Fig.42). Upon phosphorylation of CD45 at Tyr-1193 (e.g.,

by $p50^{csk}$), the SH2 domain of $P56^{lck}$ binds this site, liberating the still-phosphorylated Tyr-505, which is accessible to CD45-mediated dephosphorylation and then activate downstream of the PTKases or other proteins leading to activation or lymphocyte adhesion.

Conclusions

In summary, investigations into the involvement of CD45(R) in homotypic adhesion and T cell activation are presented. The study has lead to the following conclusions: The CD45 molecule plays an important role in lymphocyte aggregation and T cell activation. Aggregation is an active process and both LFA-1-dependent and LFA-1-independent pathways are involved. Some aggregation results from altered phosphorylation of cellular tyrosine proteins and aggregation via tyrosine phosphatase is calcium-dependent. Aggregation by the same Mab on the different cell lines is effected through different pathways. CD45RB (6B6, 6G3, MT3), CD45RO (UCHL-1, OPD4, and A-6), and CD45 (4D11, 4C9, and 2G1) Mabs can inhibit OKT3-induced T cell activation. The CD45 Mabs mainly inhibit activation of the CD8+ T cell subset. A possible mechanism for the inhibition of T cell activation involves the interference with tyrosine phosphorylation of particular proteins.

Although an understanding of the molecular basis of lymphocyte adhesion and T cell activation needs further

investigation, homotypic adhesion can nonetheless serve as a sensitive and rapid assay for examination of signals generated through cell surface molecules. The identification of the LFA-1-independent adhesion receptors will elucidate the physiologic significance of this regulatory system. Further investigation of the exact mechanisms by which CD45 Mabs mediate inhibition of T cell activation will expand our knowledge on the signal pathways and lead to improvements in immunomodulation as a way of treating graft rejection and a number of autoimmune diseases.

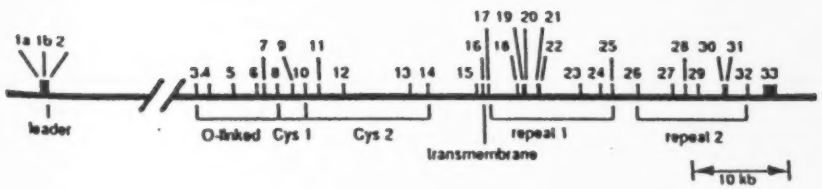

FIGURE 1. Genomic structure for the CD45 gene. Exons that encode possible subdomains are grouped and labelled below the line. The numbers above the line represent the number of exons. The size of each exon is not to scale. This Fig. is from Ref.1.

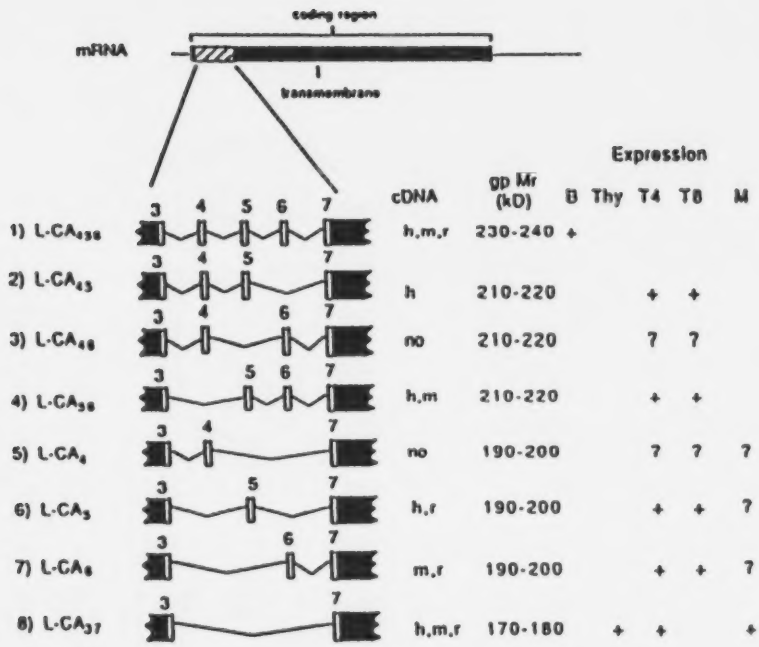

FIGURE 2. Schematic diagram of the differential exon usage for CD45 mRNA. Exons 3-7 are indicated by open rectangles. The exons used within each form are indicated by the CD45 subscript and the splicing events by the V. This Fig. is from Ref.1.

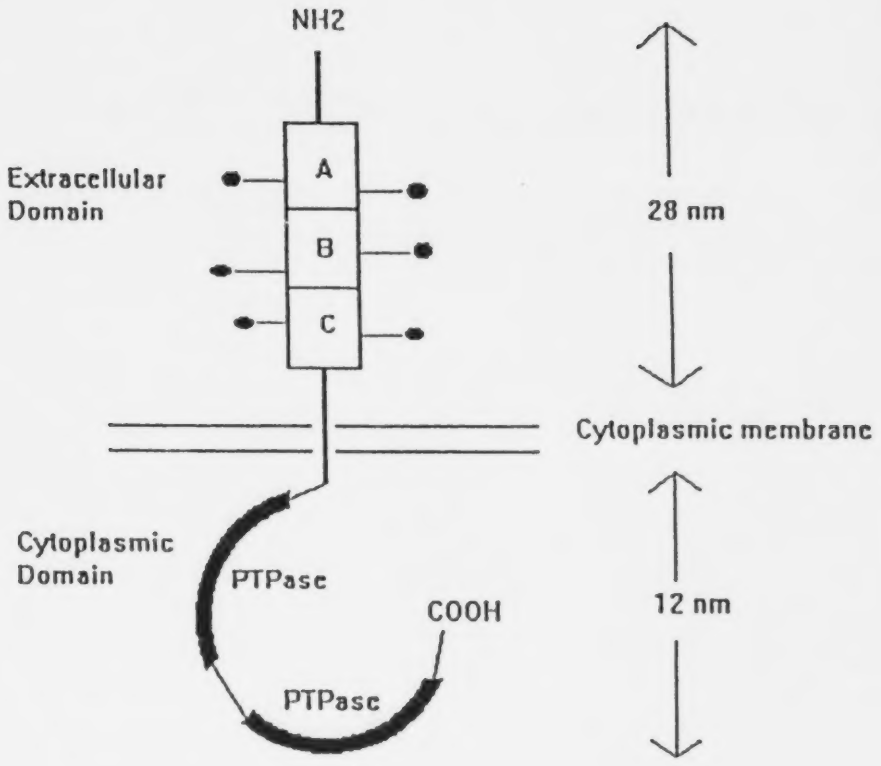

FIGURE 3. Diagram of CD45 molecule which consists of three domains, ie. Extracellular domain, Transmembrane domain, and Cytoplasmic domain which has PTPase activity.

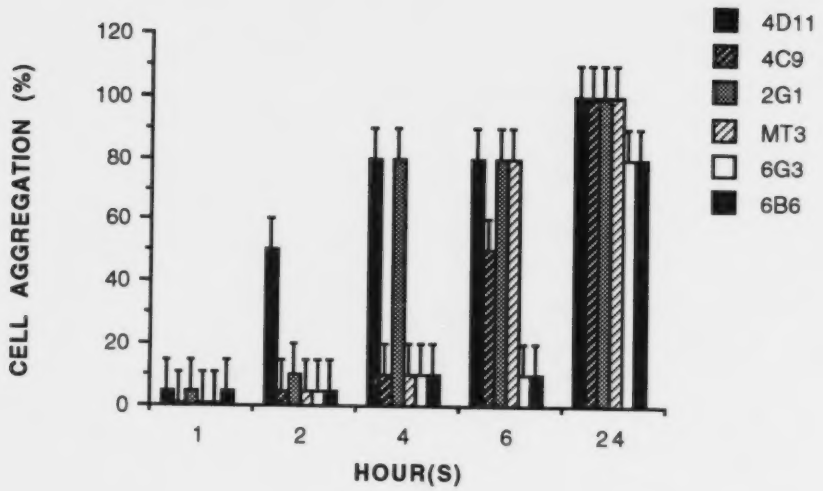

FIGURE 4. CD45 (4D11, 4C9, and 2G1) and CD45RB (MT3, 6G3, and 6B6) induced Jurkat cell line homotypic adhesion with different rate.

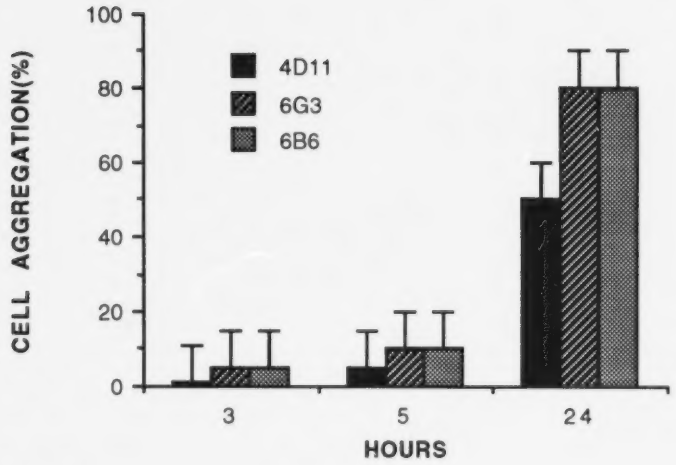

FIGURE 5. CD45 4D11 and CD45RB (6G3 and 6B6) induced Hoon cell line homotypic adhesion with different rate and becoming maximal by 24 hs.

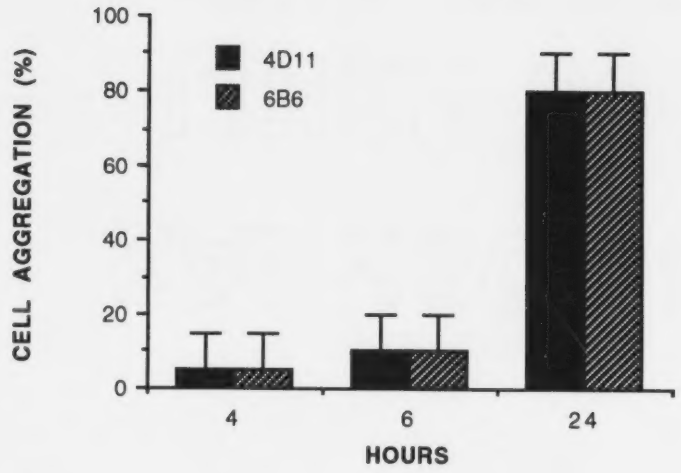

FIGURE 6. CD45 4D11 and CD45RB 6B6 mab induced Ramos cell line homotypic adhesion. Aggregation became maximal by 24 hs.

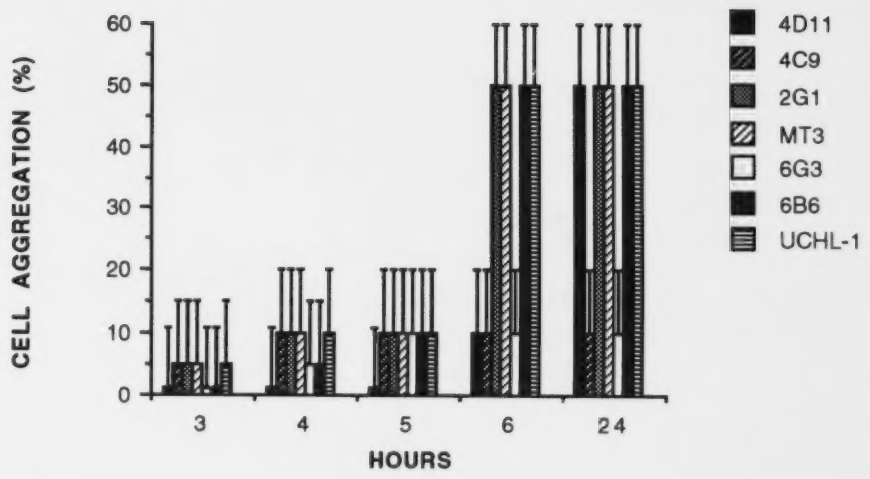

FIGURE 7. CD45 (4D11, 4C9, and 2G1), CD45RB (MT3, 6G3, and 6B6), and CD45RO UCHL-1 Mabs induced Rose cell line homotypic adhesion with different rate.

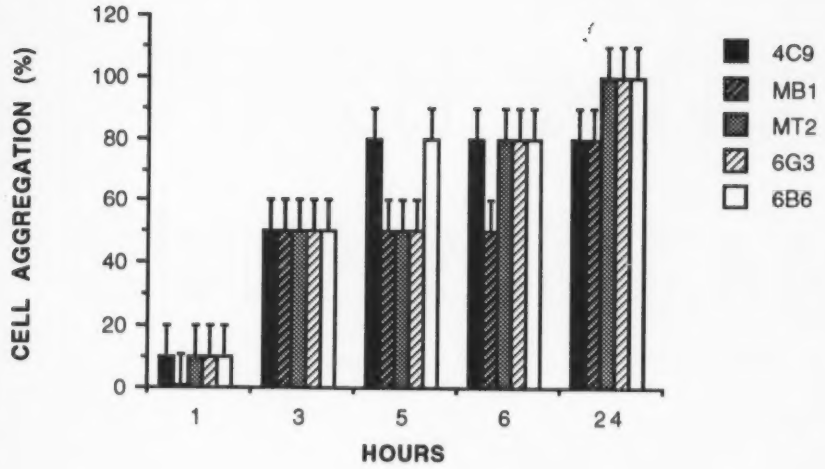

FIGURE 8. CD45 4C9, CD45RA (MB1 and MT2), and CD45RB (6G3 and 6B6) Mabs induced KG1a cell line homotypic adhesion with different rate.

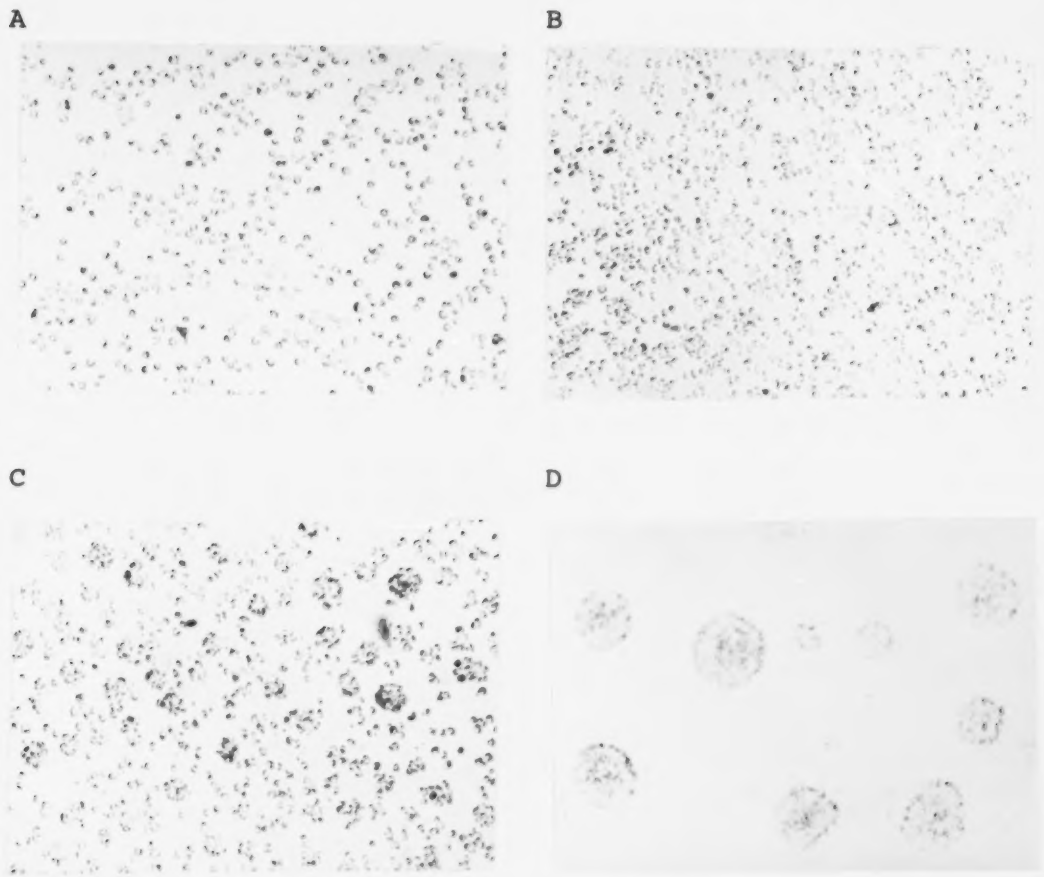

FIGURE 9. A semi-quantitative scoring of homotypic aggregation-induced by different CD45 Mabs. The stem cell line KG1a was utilized in homotypic aggregation induced by CD45 4C9 Mab. (A) no aggregation was induced at 30 min (0); (B) aggregation was induced at 1 hr (1+); (C) aggregation was induced at 5 hr (3+); (D) aggregation was induced at 24 hr (4+).

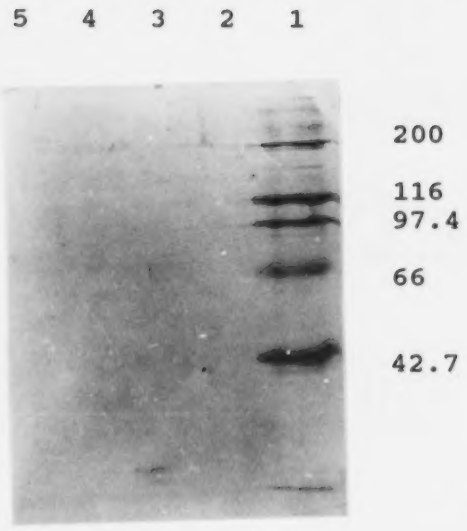

FIGURE 10. Identification of phosphotyrosine-containing proteins in Molt4 cell homotypic aggregation induced by CD45RB 6B6 Mab stained with 4G10. 1. Standard 2. Molt4 cells alone 3. Molt4 Cells+6B6 4. Molt4 cells+6B6+vanadate 5. Molt4 cells + 4D11 (for negative control).

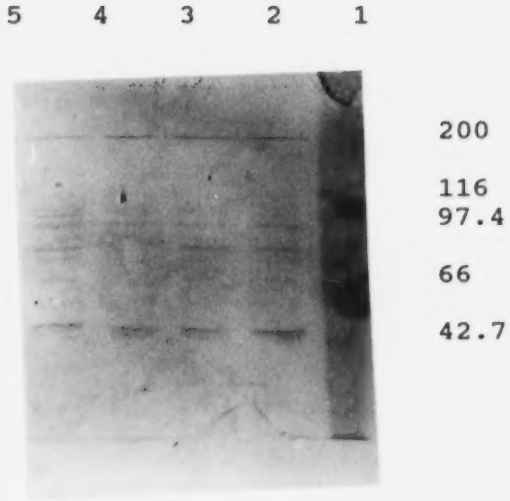

FIGURE 11. Identification of phosphotyrosine-containing proteins in Hoon cell homotypic aggregation induced by CD45 4D11, CD45RB 6G3 and 6B6 Mabs stained with 4G10. 1. Standard 2. Hoon cells alone 3. Hoon cells+4D11 4. Hoon cells+4D11+ vanadate 5. Hoon cells+6G3.

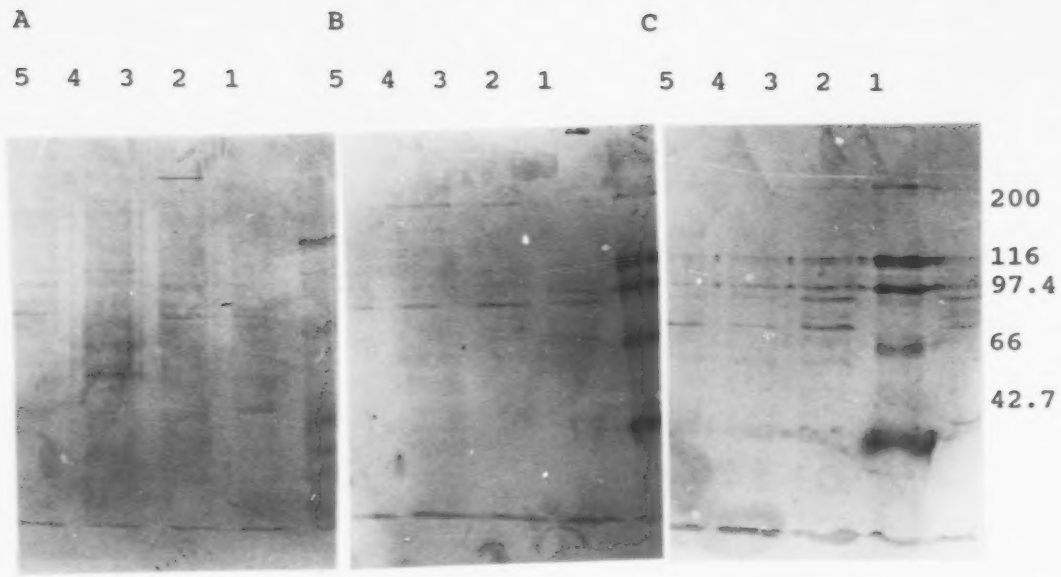

FIGURE 12. Identification of phosphotyrosine-containing proteins in Jurkat cell homotypic aggregation induced by CD45RB Mabs (6B6, 6G3, and MT3), CD45 Mabs (4D1, 4C9, and 2G1) stained with 4G10. (A): 1. Standard 2. Jurkat cells 3. Jurkat cells+MT3 4. Jurkat cells+MT3+vanadate 5. Jurkat cells+6B6. (B): 1. Standard 2. Jurkat cells 3. Jurkat cells+6G3 4. Jurkat cells+6G3+vanadate 5. Jurkat+2G1. (C): 1. Jurkat cells 2. Standard 3. Jurkat cells+4C9 4. Jurkat cells+4C9+vanadate 5. Jurkat cells+4C9.

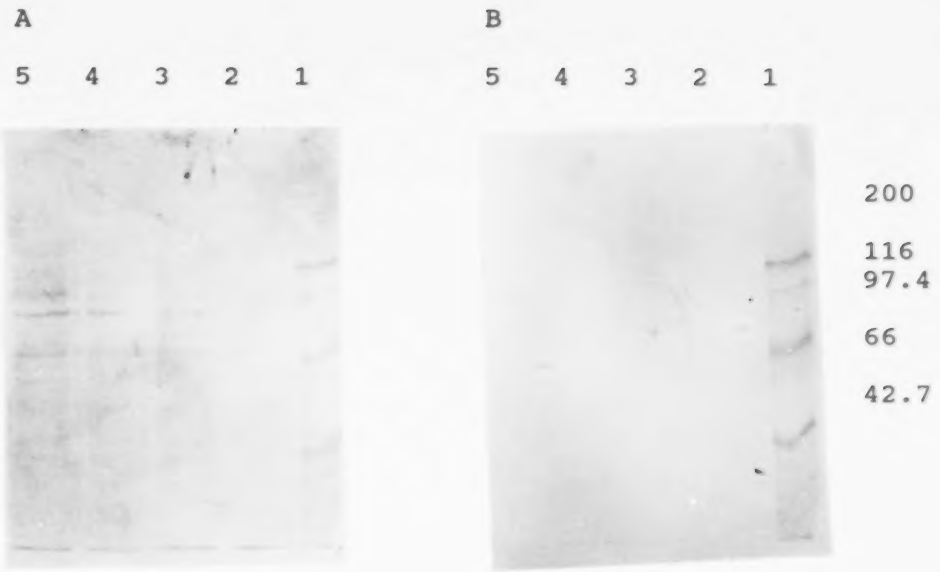

FIGURE 13. Identification of phosphotyrosine-containing proteins in KG1a cell homotypic aggregation induced by CD45 4C9, CD45RB 6B6 and 6G3, and CD45RA MB1 and MT2 Mabs stained with 4G10. (A) 1. Standard 2. KG1a cells alone 3. KG1a+4C9 4. KG1a +MT2 5. KG1a+6G3 (B) 1. Standard 2. KG1a cells alone 3. KG1a cells+6B6 4.KG1a cells+MB1 5.KG1a+MB1+Vanadate.

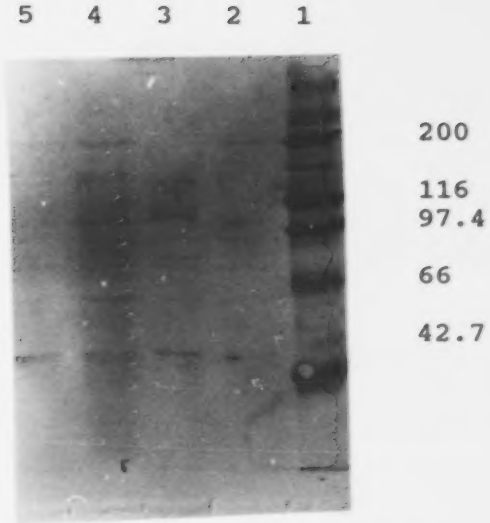

FIGURE 14. Identification of phosphotyrosine-containing proteins in Rose cell homotypic aggregation induced by CD45RO UCHL-1 and CD45RB 6G3 Mabs stained with 4G10. 1. Standard 2. Rose cells alone 3. Rose cells+6G3 4. Rose cells+ 6G3+ vanadate 5. Rose cells+UCHL-1.

TABLE 1: HOMOTYPIC AGGREGATION INDUCED BY CD45 Mabs ON CELL LINES [a]

Cell lines	4D11	4C9	2G1	MB1	MT2	MT3	6G3	6B6	UCHL-1
T cell lines									
Jurkat	4	4	4	0	0	4	3	3	0
Molt4	0	0	0	0	0	0	0	3	0
Pre-B cell lines									
Nalm 6	0	0	0	0	0	0	0	0	0
Hoon	2	0	0	0	0	0	4	4	0
B cell lines									
Ramos	3	0	0	0	0	0	0	3	0
Rose	2	2	3	0	0	4	4	4	4
Daudi	0	0	0	0	0	0	0	0	0
VER	0	0	0	0	0	0	0	0	0
ZYL	0	0	0	0	0	0	0	0	0
Raji	0	0	0	0	0	0	0	0	0
Schi	0	0	0	0	0	0	0	0	0
Stem cell line									
KGla	0	3	0	3	4	0	4	4	0
ALCL cell line									
Karpas	0	0	0	0	0	0	0	0	0
Hodgkin's cell lines									
L428	0	0	0	0	0	0	0	0	0
DEV	0	0	0	0	0	0	0	0	0

a. Homotypic adhesion assays were performed with different CD45 and CD45R Mabs as described in Materials and Methods. Results were obtained at 24 hs.

TABLE 2: CALCIUM INVOLVEMENT IN CD45-INDUCED HOMOTYPIC AGGREGATION [a]

Cell lines	4D11	4C9	2G1	MB1	MT2	MT3	6G3	6B6	UCHL-1
T cell lines									
Jurkat	- [b]	+ [c]	-			+	+	-	
Molt4								+	
Pre-B cell line									
Hoon	+						-	-	
B cell lines									
Ramos	+							-	
Rose	-	+	-			+	+	+	+
Stem cell line									
KG1a		+		+	-		-	-	

a. Homotypic aggregation assays were performed as described in Materials and Methods.

b. - indicated that verapamil (10uMol/ml)did not inhibit homotypic aggregation induced by different CD45 and CD45R Mabs.

c.+ indicated that verapamli (10uMol/ml) inhibited homotypic aggregation induced by different CD45 and CD45R Mabs.

TABLE 3: LFA-1/ICAM-1 INVOLVING CD45 Mab-induced AGGREGATION [a]

Cell lines	4C9	MB1	MT2	6G3	6B6
T cell line					
Molt4					+ [b]
Stem cell line					
KG1a	+	+	+	- [c]	-

a. Homotypic aggregation assays were performed as described in Materials and Methods.

b. + indicated that CD18 Mab (25ug/ml) inhibited homotypic aggregation induced by CD45 and CD45R MAbs.

c. - indicated that CD18 Mab did not inhibit homotypic aggregation by CD45 and CD45R Mabs.

TABLE 4: TYROSINE PHOSPHATASE INVOLVED IN CD45-induced HOMOTYPIC AGGREGATION[a]

Cell lines	4D11	4C9	2G1	MB1	MT2	MT3	6G3	6B6	UCHL-1
T cell lines									
Jurkat	-[b]	+[c]	-			+	+	-	
Molt4								+	
Pre-B cell line									
Hoon		+					-	-	
B cell lines									
Ramos		-						-	
Rose		-	+	-		-	+	+	-
Stem cell line									
KG1a		-		+	-		-	-	

a. Homotypic aggregation assays were performed as describe in Material and Methods.

b. - indicated that vanadate(20uMlo/ml) did not inhibit homotypic aggregation induced by CD45 and CD45R Mabs.

c. + indicated that vanadate (20uMol/ml) inhibited homotypic aggregation induced by CD45 and CD45R Mabs.

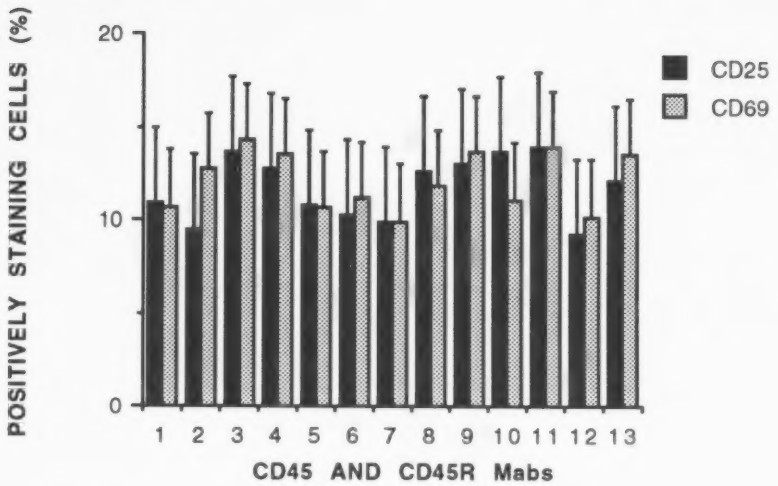

FIGURE 15. None of the CD45 and CD45R Mabs alone were able to affect both CD25 and CD69 expression on T cells (different CD45 or CD45R Mabs vs. media alone $P>0.05$). 1. control 2. 6B6 3. 6G3 4. MT3 5. 4D11 6. 4C9 7. 2G1 8. MB1 9. MT2 10. UCHL-1 11. 4F9 12. 6B6+UCHL-1 13. OPD4. Values represent the mean (± SD) percentage of inhibition or stimulation.

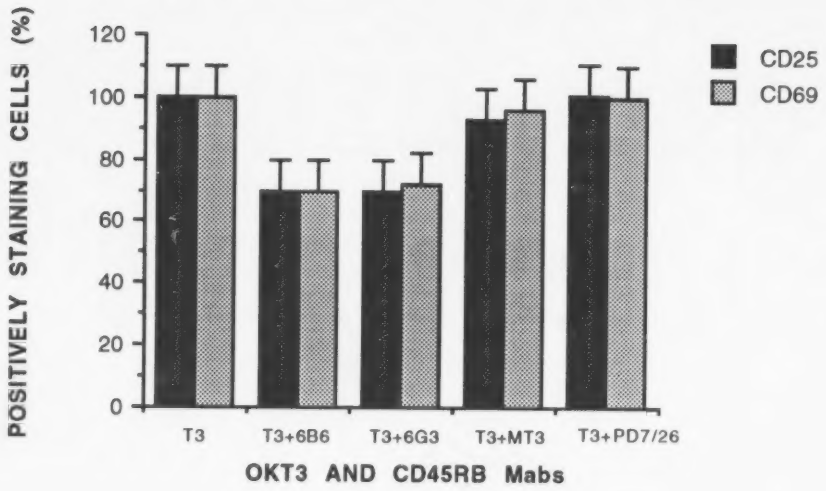

FIGURE 16. CD45RB (6B6, 6G3, and MT3) Mabs were able to inhibit OKT3-induced T cell activation. While PD7/26 has no effect on T cell activation (PD7/26 vs. OKT3 alone $P>0.05$) . 6B6, 6G3, and MT3 vs. OKT3 alone for both CD25 and CD69 staining ($P<0.05$). Values represent the mean (± SD) percentage of inhibition.

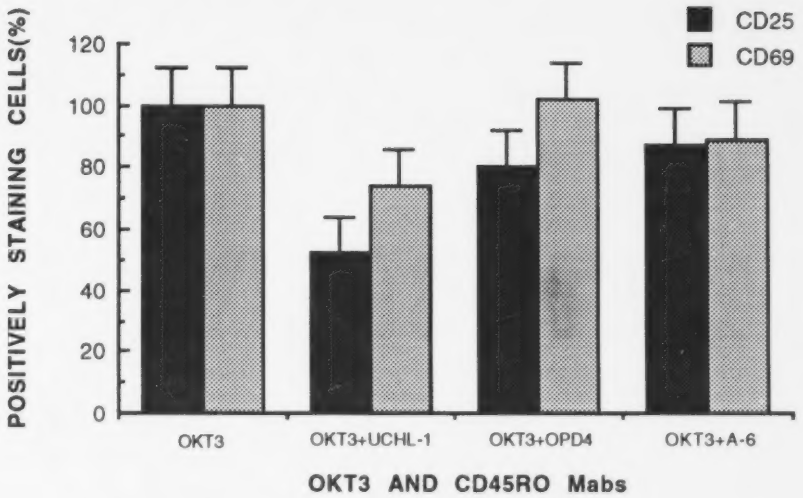

FIGURE 17. CD45RO (UCHL-1, OPD4, and A-6) Mabs were capable of blocking OKT3-induced CD25 and CD69 expression (those Mabs vs. OKT3 alone $P<0.05$) except OPD4 on CD69 expression ($P>0.05$). Among them, UCHL-1 was the strongest inhibitor. UCHL-1 vs.A-6 and OPD4 vs. A-6 ($P<0.05$) for CD25 staining. UCHL-1 vs. OPD4 and UCHL-1 vs. A-6 ($P<0.05$) for CD69 staining. Values represent the mean (± SD) percentage of inhibition.

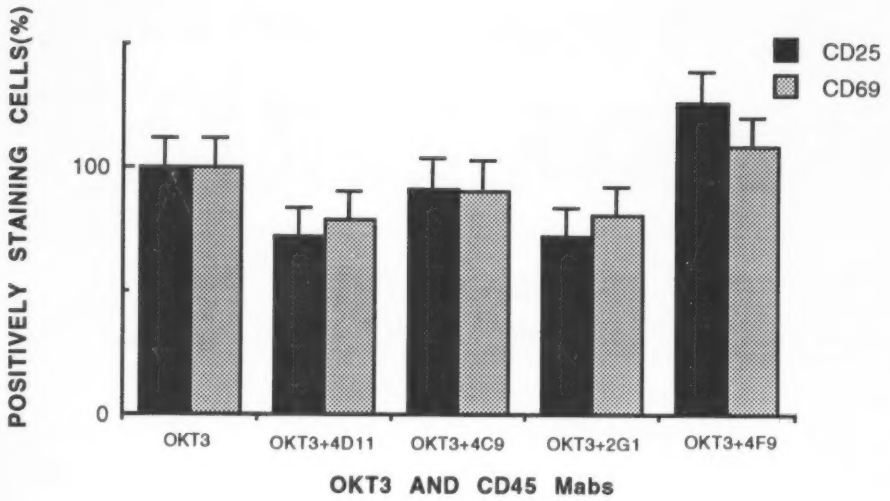

FIGURE 18. CD45 (4D11, 4C9, and 2G1) Mabs were able to inhibit OKT3-induced T cell activation (those Mabs vs. OKT3 alone $P<0.05$) for both CD25 and CD69 staining. While CD45 4F9 Mab can stimulate T cell activation (4F9 vs. OKT3 $P<0.05$). 4D11 vs. 4C9 ($P<0.05$) for both CD25 and CD69 staining. Values represent the mean (± SD) percentage of inhibition or stimulation.

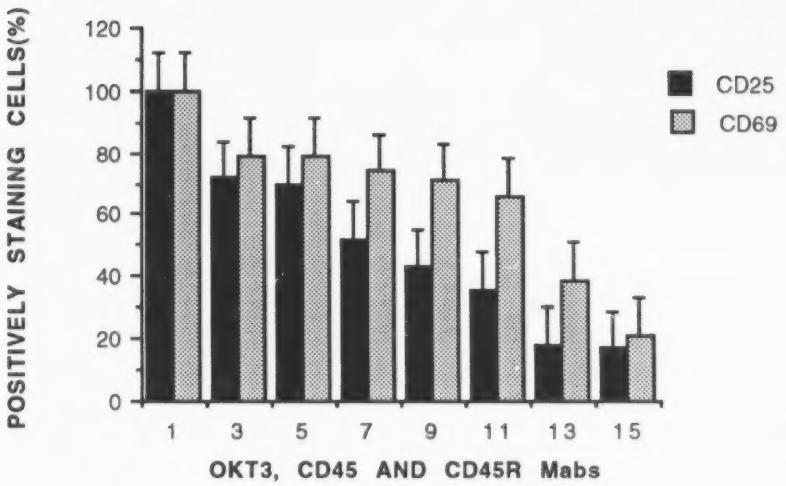

FIGURE 19. CD45 4D11 Mab , CD45RB 6G3 Mab plus CD45RO UCHL-1 Mab could synergically inhibit OKT3-induced T cell activation ($P<0.05$). 6G3, 4D11 plus UCHL-1 were able to completely inhibit OKT3-induced CD25 expression on T cells comparing with control. 1. OKT3 3. OKT3+4D11 5. OKT3+6G3 7. OKT3+UCHL-1 9. OKT3+4D11+UCHL-1 11. OKT3+6G3+UCHL-1 13. OKT3+6G3+4D11+ UCHL-1 15. Control. Values represent the mean (± SD) percentage of inhibition.

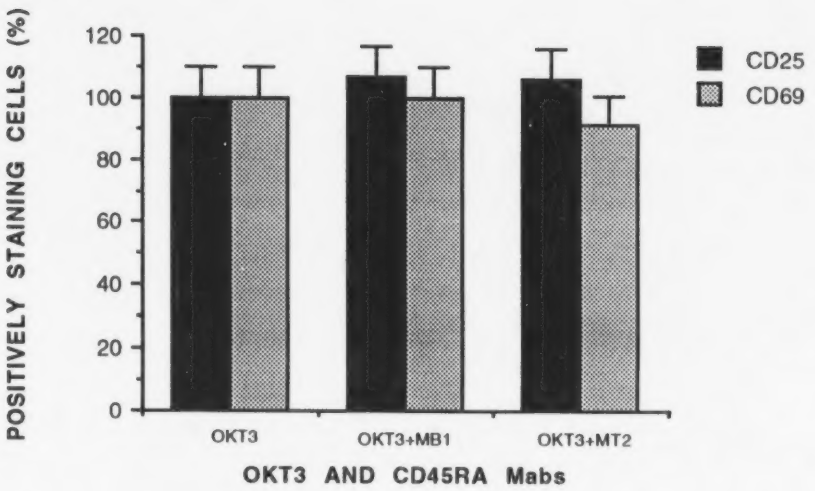

FIGURE 20. CD45RA MT2 Mab had no effects on OKT3-induced T cell activation (MT2 vs. OKT3 alone $P>0.05$). While MB1 had a costimulatory effect on OKT3-induced T cell activation (MB1 vs. OKT3 $P<0.05$) for CD25 staining. Values represent the mean (± SD) percentage of inhibition or stimulation.

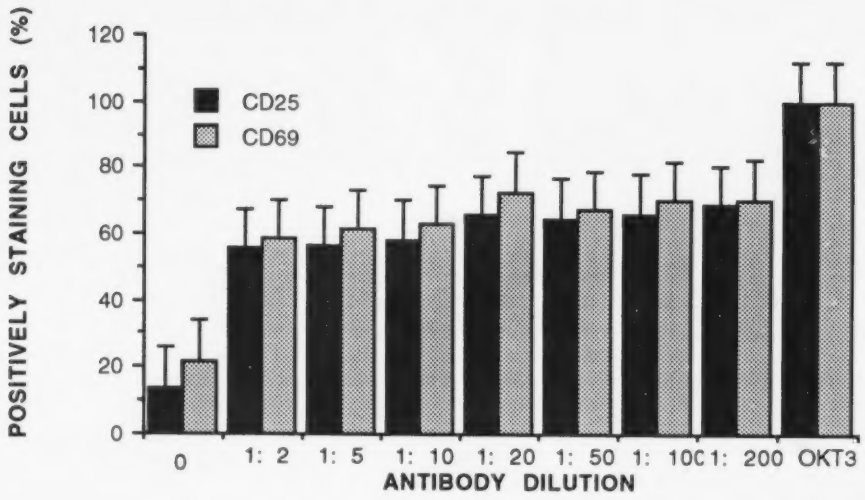

FIGURE 21. CD45RB 6G3 Mab could inhibit OKT3-induced T cell activation in a dose-dependent fashion up to a concentration 2-5ug/ml with no further significant increase in inhibition at 10-25ug/ml. Values represent the mean (± SD) percentage of inhibition.

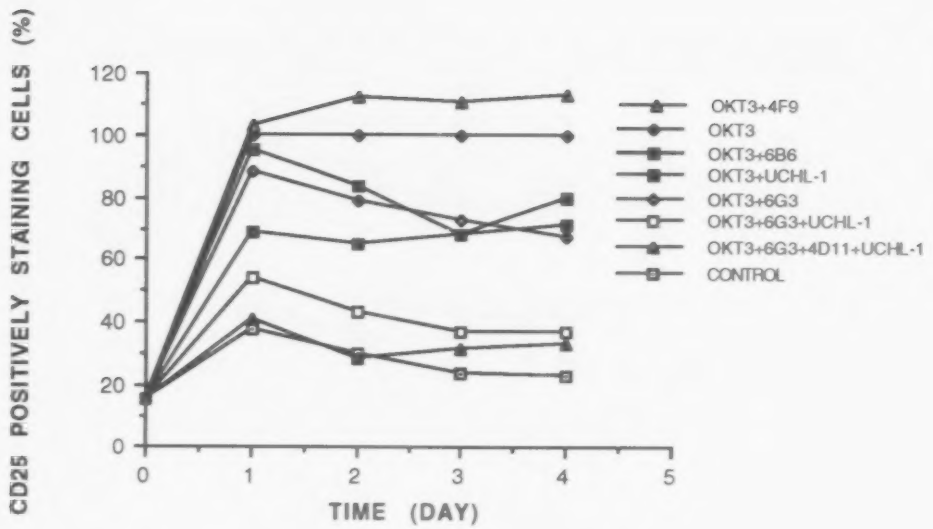

FIGURE 22. The kinetics experiments were shown that UCHL-1 consistently inhibit OKT3-induced T cell activation from day 1 to day 4, where 6B6 and 6G3 (CD45RB) Mab block OKT3-induced T cell activation from day 2 and significantly inhibited T cell activation after day 3. Further, 6G3 plus UCHL-1 and 4D11, 6G3 plus UCHL-1 were able block T cell activation from day 1. 6G3, 4D11 plus UCHL-1 completely inhibited OKT3-induced T cell activation. In contrast, CD45 4F9 Mab could co-stimulate T cell activation with OKT3. All data shown were being gated on CD25 expression. Values represent the mean (± SD) percentage of inhibition or stimulation.

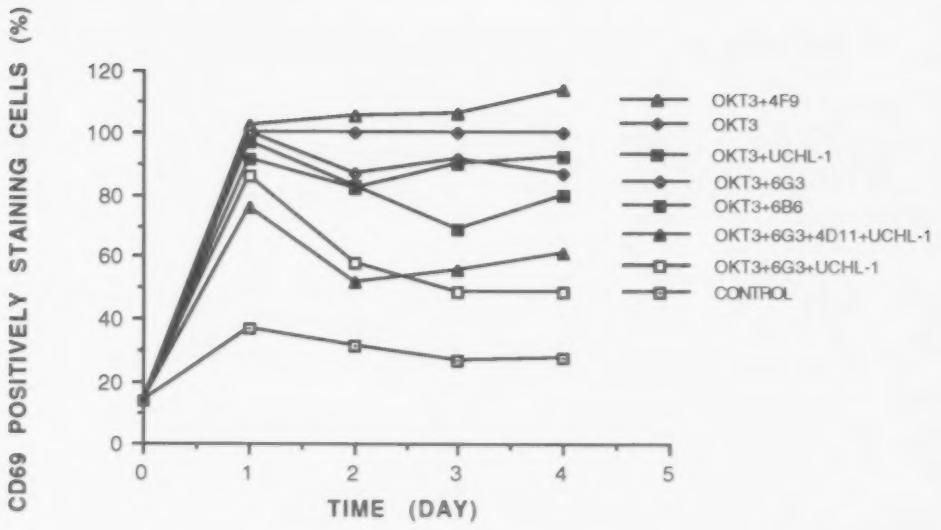

FIGURE 23. CD45 and CD45R Mabs showed significantly inhibition of OKT3-induced T cell activation on CD69 expression after day 2 except that 6G3, 4D11 plus UCHL-1 exerted its blocking effect after day 1. In contrast, CD45 4F9 Mab had a co-stimulation effect on OKT3-induced T cell activation. Values represent the mean (± SD) percentage of inhibition or stimulation.

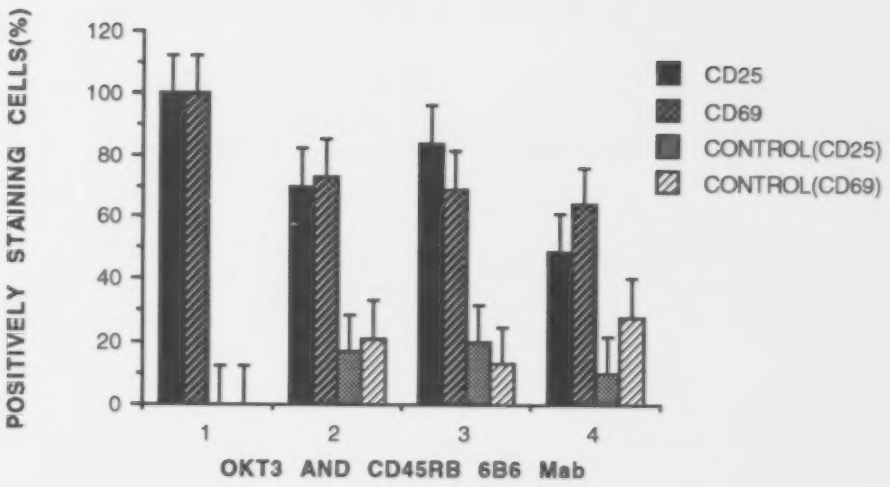

FIGURE 24. CD45RB 6B6 Mab mainly inhibited the CD8+ T cell subset in OKT3-induced T cell activation on CD25 expression (CD4+ vs, CD8+ T cells $P<0.05$) but not CD69 expression ($P>0.05$). 1. OKT3 2. OKT3+6B6 3.OKT3+6B6 (gating CD4 positively staining T cells) 4. OKT3+6B6 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of inhibition.

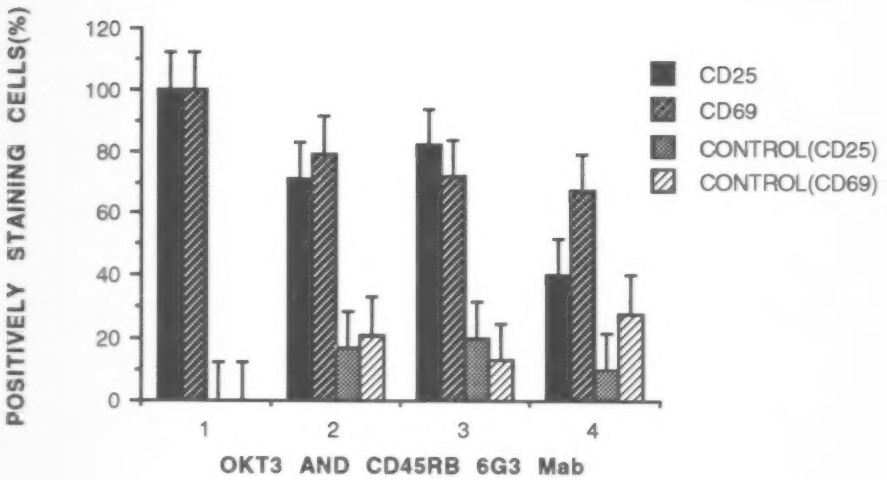

FIGURE 25. CD45RB 6G3 Mab mainly inhibited CD8+ T cell subset in OKT3-induced T cell activation on CD25 expression (CD4+ vs. CD8+ T cells $P<0.05$). 1. OKT3 2. OKT3+6G3 3. OKT3+6G3 (gating CD4+positively staining T cells) 4. OKT3+6G3 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of inhibition.

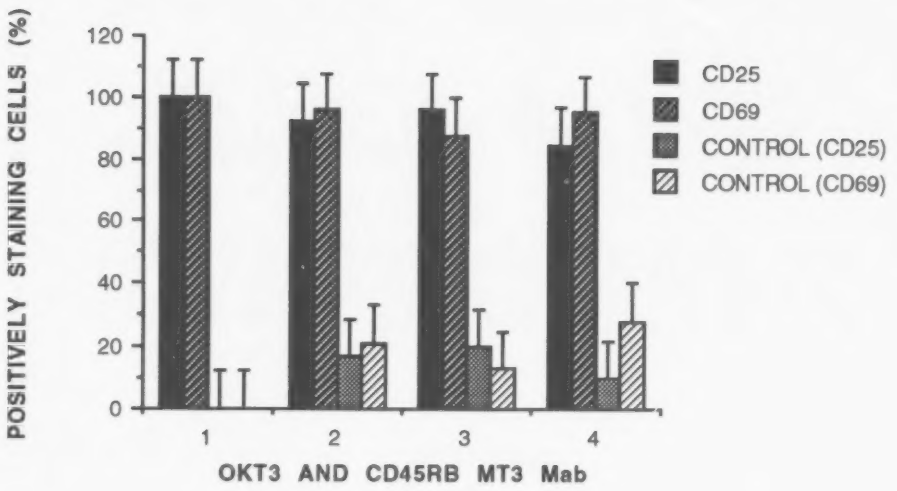

FIGURE 26. CD45RB MT3 mainly inhibited CD8+ T cell subset in OKT3-induced T cell activation on CD25 expression (CD4+ vs. CD8+ T cells $P<0.05$). 1. OKT3 2. OKT3+MT3 3. OKT3+MT3 (gating CD4+ positively staining T cells) 4. OKT3+MT3 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of inhibition.

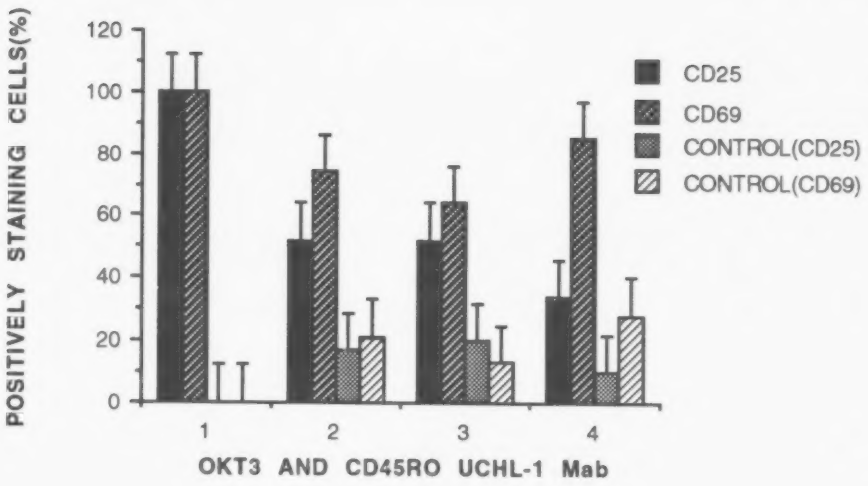

FIGURE 27. CD45RO UCHL-1 Mab mainly inhibited CD8+ T cell subset in OKT3-induced T cell activation on CD25 expression (CD4+ vs. CD8+ T cells $P<0.05$) and inhibited CD4+ T cell subset on CD69 expression (CD4+ vs. CD8+ T cells $P<0.05$). 1. OKT3 2. OKT3+UCHL-1 3. OKT3+UCHL-1 (gating CD4+ positively staining T cells) 4. OKT3+UCHL-1 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of inhibition.

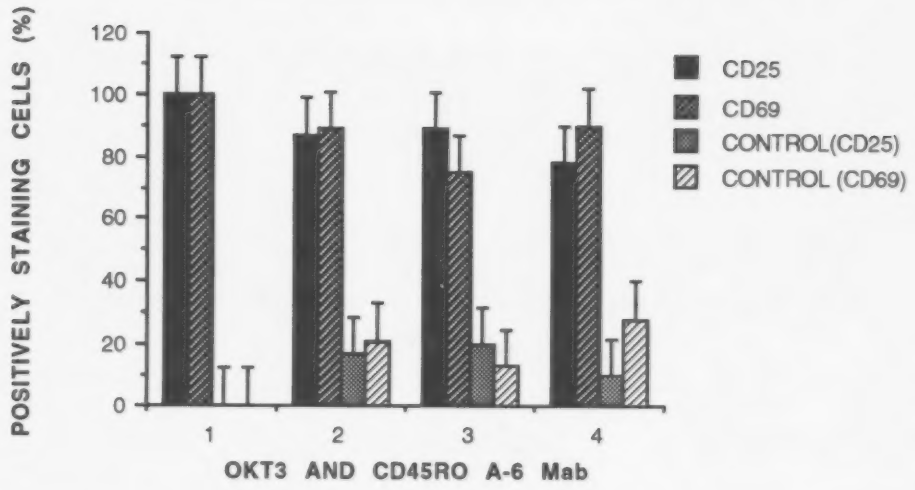

FIGURE 28. CD45RO A-6 Mab mainly inhibited CD8+ T cell subset in OKT3-induced T cell activation on CD25 expression (CD4+ vs. CD8+ T cells $P<0.05$) but not on CD69 expression. 1. OKT3 2. OKT3+A-6 3. OKT3+A-6 (gating CD4+ positively staining T cells) 4. OKT3+A-6 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of inhibition.

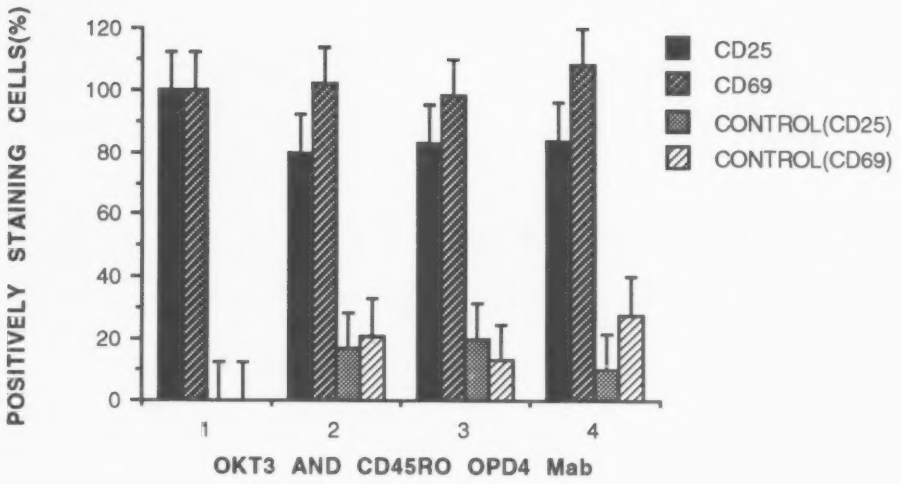

FIGURE 29. CD45RO OPD4 Mab mainly inhibited CD4+ T cell subset on CD69 expression (CD4+ vs. CD8+ T cells $P<0.05$) but not CD25 expression on both CD4+ and CD8+ T cells ($P>0.05$). 1. OKT3 2. OKT3+OPD4 3. OKT3+OPD4 (gating CD4+ positively staining T cells) 4. OKT3+OPD4 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of inhibition.

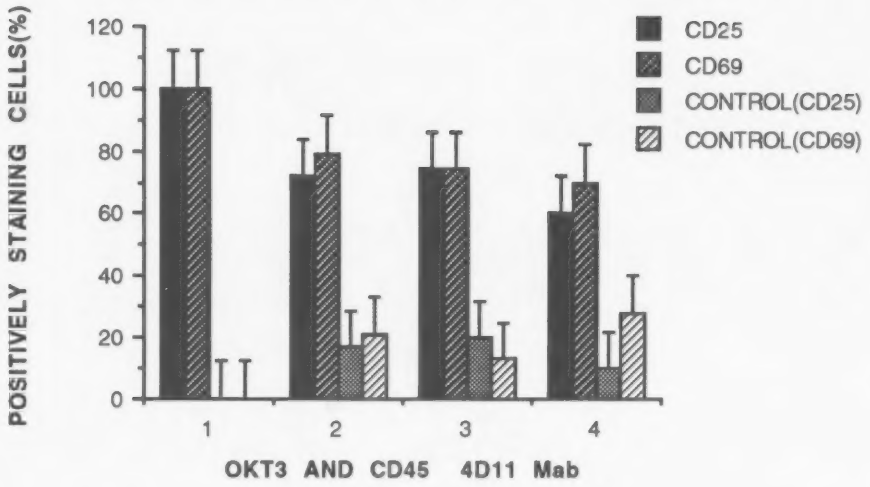

FIGURE 30. CD45 4D11 Mab inhibited both CD4+ and CD8+ T cell subsets in OKT3-induced T cell activation on both CD25 and CD69 expressions (CD4+ vs. CD8+ $P>0.05$). 1. OKT3 2. OKT3+4D11 3. OKT3+4D11 (gating CD4+ positively staining T cells) 4. OKT3+4D11 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of inhibition.

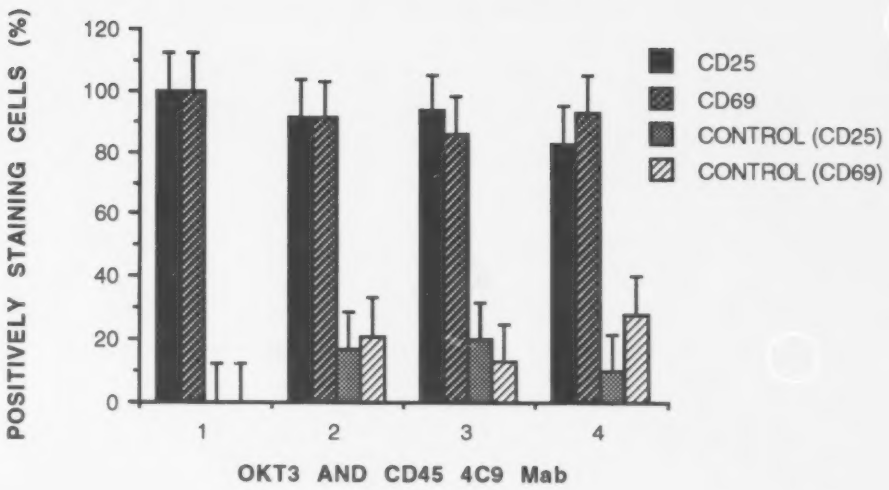

FIGURE 31. CD45 4C9 Mab inhibited CD8+ T cell subsets on CD25 expression (CD4+ vs. CD8+ T cells $P<0.05$) and both CD4+ and CD8+ T cells on CD69 expression (CD4+ vs. CD8+ T cells $P>0.05$). 1. OKT3 2. OKT3+4C9 3. OKT3+4C9 (gating CD4+ positively staining T cells) 4. OKT3+4C9 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of inhibition.

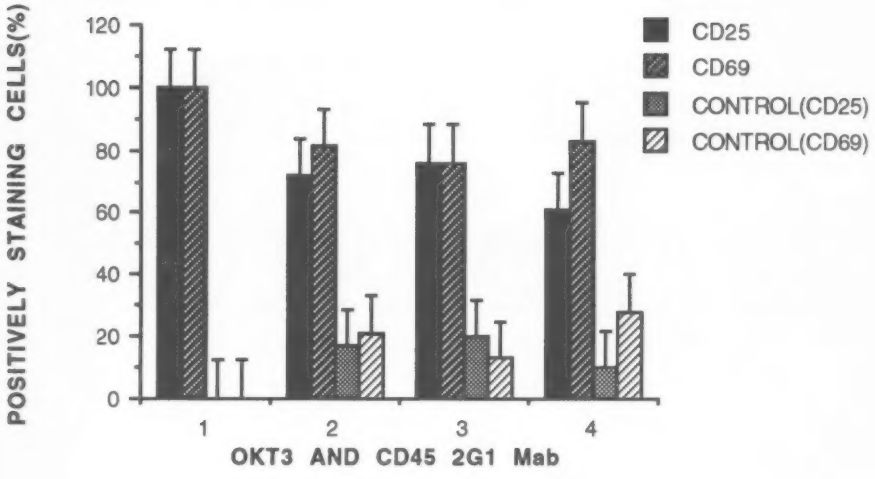

FIGURE 32. CD45 2G1 Mab mainly inhibited CD8+ T cell subset on CD25 expression (CD4+ vs. CD8+ T cells $P<0.05$) but not on CD69 expression. 1. OKT3 2. OKT3+2G1 3. OKT3+2G1 (gating CD4+ positively staining T cells) 4. OKT3+2G1 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of inhibition.

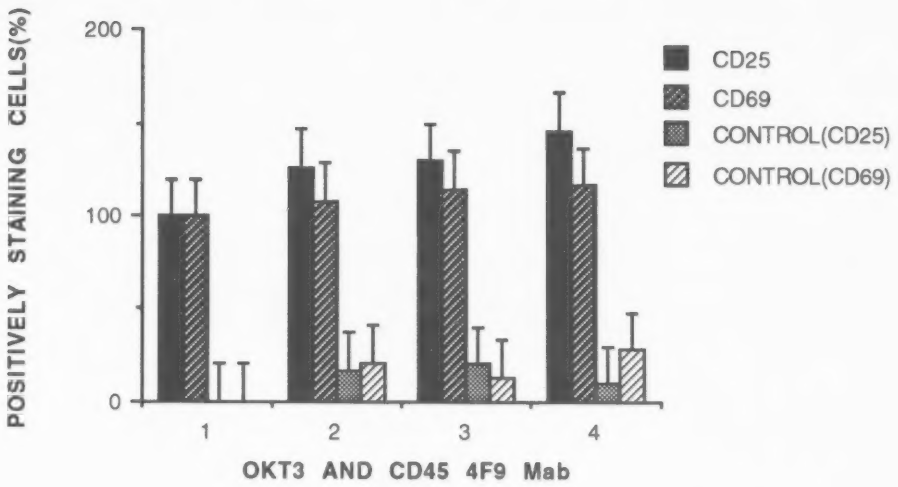

FIGURE 33. CD45 4F9 Mab co-stimulated both CD4+ and CD8+ T cell subsets on both CD25 and CD69 expressions (CD4+ vs. CD8+ $P>0.05$). 1. OKT3 2. OKT3+4F9 3. OKT3+4F9 (gating CD4+ positively staining T cells) 4. OKT3+4F9 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of stimulation.

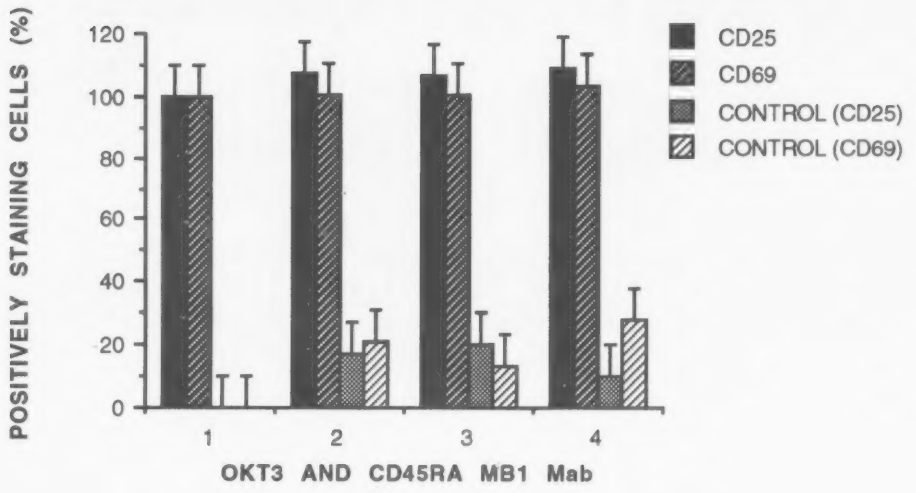

FIGURE 34. CD45RA MB1 co-stimulated both CD4+ and CD8+ T cell subsets on both CD25 and CD69 expressions (CD4+ vs. CD8+ T cells $P>0.05$). Values represent the mean (± SD) percentage of stimulation.

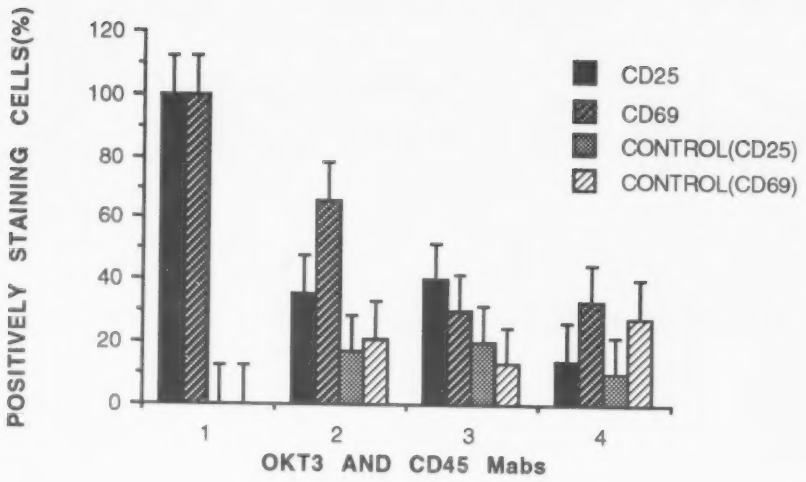

FIGURE 35. CD45RB 6G3 plus CD45RO UCHL-1 Mabs mainly inhibited CD8+ T cell subset in OKT3-induced T cell activation on CD25 expression (CD4+ vs. CD8+ T cells $P<0.05$) but not on CD69 expression. 1. OKT3 2. OKT3+6G3+UCHL-1 3. OKT3+6G3+UCHL-1 (gating CD4+ positively staining T cells) 4. OKT3+6G3+UCHL-1 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of inhibition.

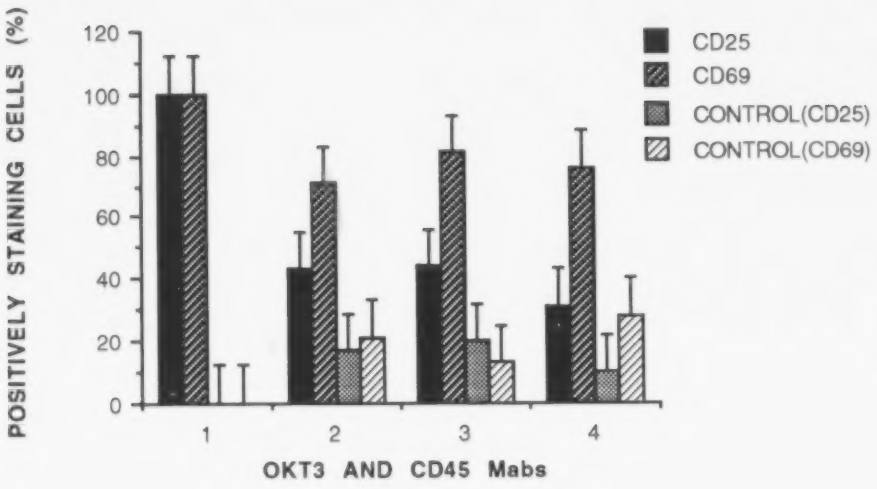

FIGURE 36. CD45 4D11 plus CD45RO UCHL-1 Mabs mainly inhibited CD8+ T cell subset on CD25 expression (CD4+ vs. CD8+ T cells $P<0.05$) but not CD69 expression. 1. OKT3 2. OKT3+4D11+UCHL-1 3. OKT3+4D11+UCHL-1 (gating CD4+ positively staining T cells) 4. OKT3+4D11+UCHL-1 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of inhibition.

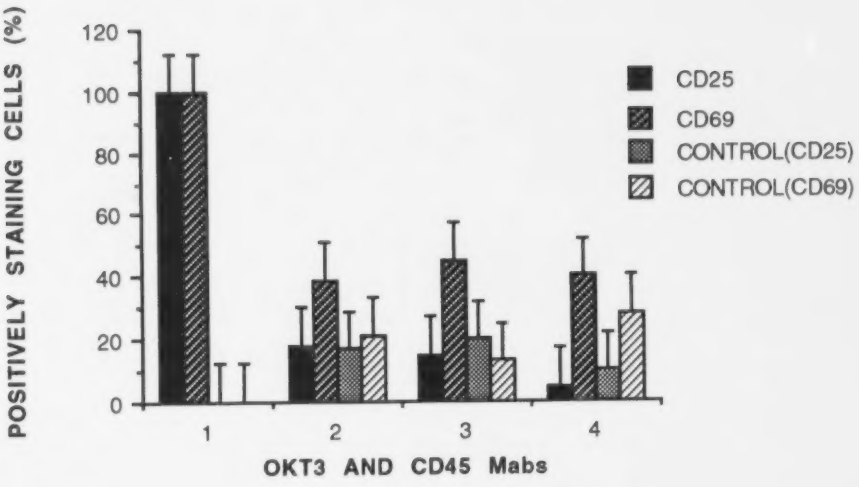

FIGURE 37. CD45RB 6G3 and CD45 4D11 plus CD45RO UCHL-1 Mabs strongly inhibited both CD4+ and CD8+ T cell subsets on CD25 expression (CD4+ vs. CD8+ T cells $P<0.05$) but not CD69 expression. 1. OKT3 2. OKT3+6G3+4D11+UCHL-1 3. OKT3+6G3+4D11+UCHL-1 (gating CD4+ positively staining T cells) 4. OKT3+6G3+4D11+UCHL-1 (gating CD8+ positively staining T cells). Values represent the mean (± SD) percentage of inhibition.

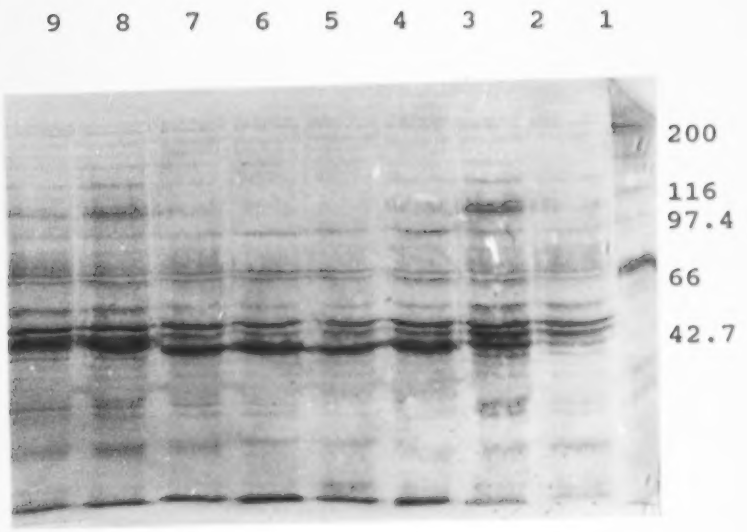

FIGURE 38. Western blots of lysates prepared from peripheral blood lymphocytes stained with 4G10. 1. Standard 2. MNC alone 3. MNC+OKT3 4.MNC+OKT3+6B6 5. MNC+6B6 6. MNC+OKT3+6G3 7. MNC+6G3 8. MNC+OKT3+MT3 9. MNC+MT3. 100 KDa protein was phosphorylated in MNC stimulated with OKT3 after 10 min and disappeared with adding CD45RB 6B6 and 6G3 Mabs. After adding CD45RB MT3 Mab, the intensity of this band was decreased.

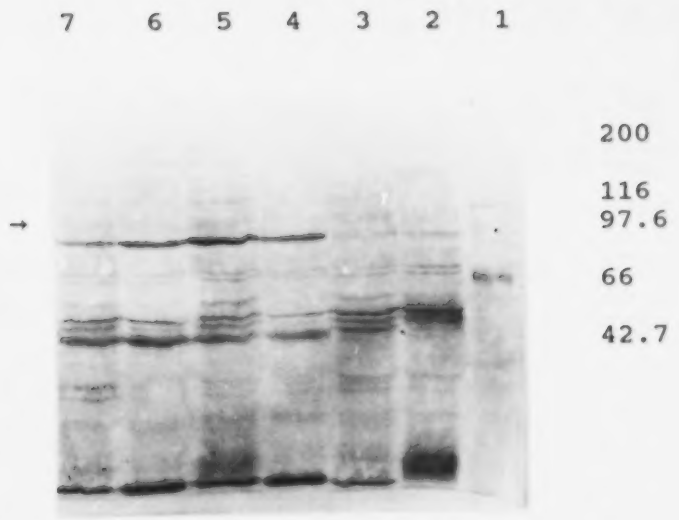

FIGURE 39. Western blots of lysates prepared from peripheral blood lymphocytes stained with 4G10. 1. Standard 2. MNC alone 3. MNC+OKT3 4. MNC+OKT3+UCHL-1 5. MNC+UCHL-1 6. MNC+OKT3+ 4F9 7. MNC+4F9. 100 KDa protein was phosphorylated in MNC stimulated with OKT3 after 10 min and disappeared with adding CD45RO UCHL-1 Mab but not CD45 4F9 Mab.

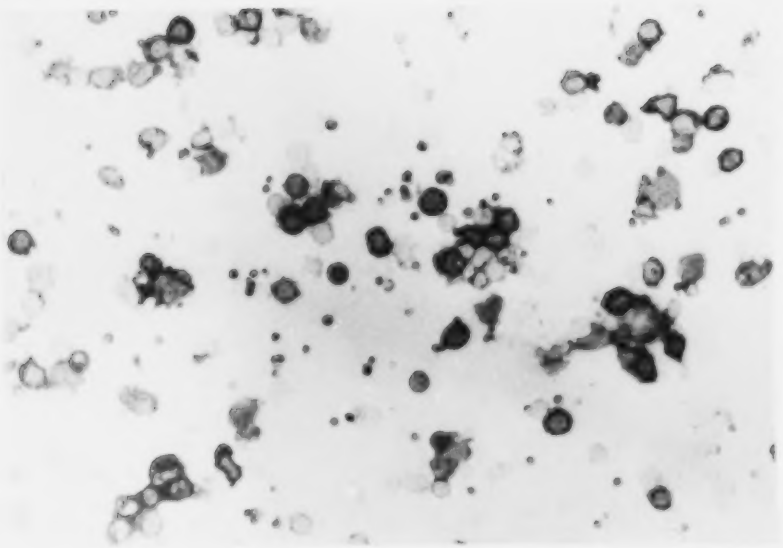

FIGURE 40. Immunoperoxidase stained MNC slides prepared from peripheral blood stained with 4G10. Note positive staining after pretreatment of MNC with vanadate 1 min followed by OKT3 for 5 min.

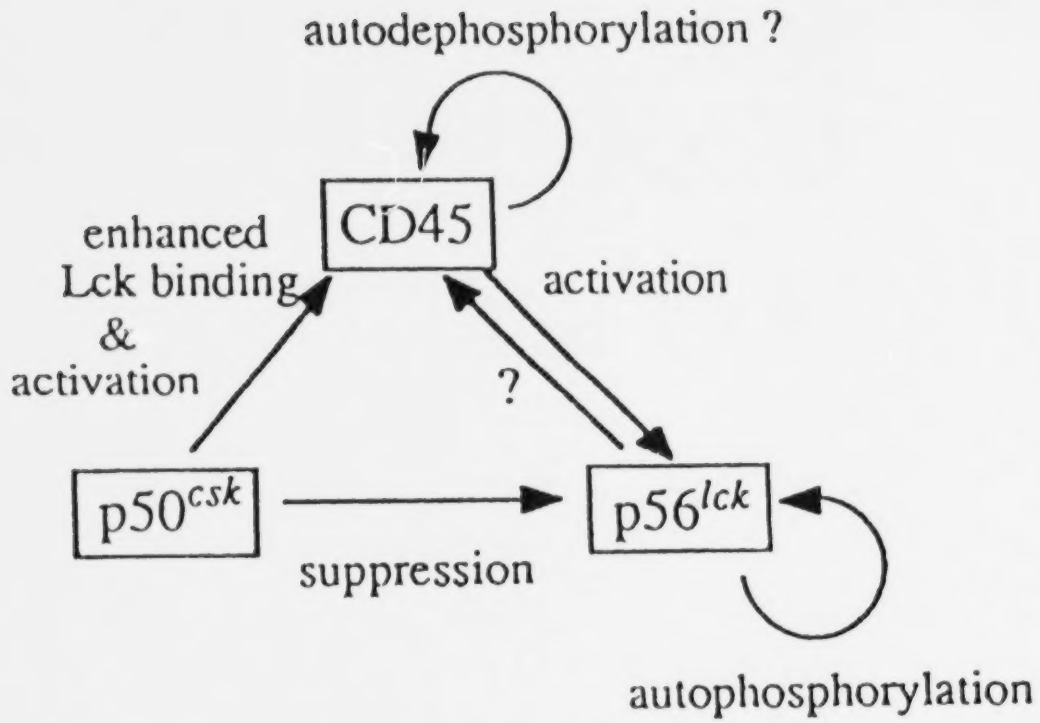

FIGURE 41. Schematic presentation of the interactions in the CD45-p50^{csk}-p56^{lck} triangle. Arrows denote phosphorylation or dephosphorylation. This Figure is from Ref.24.

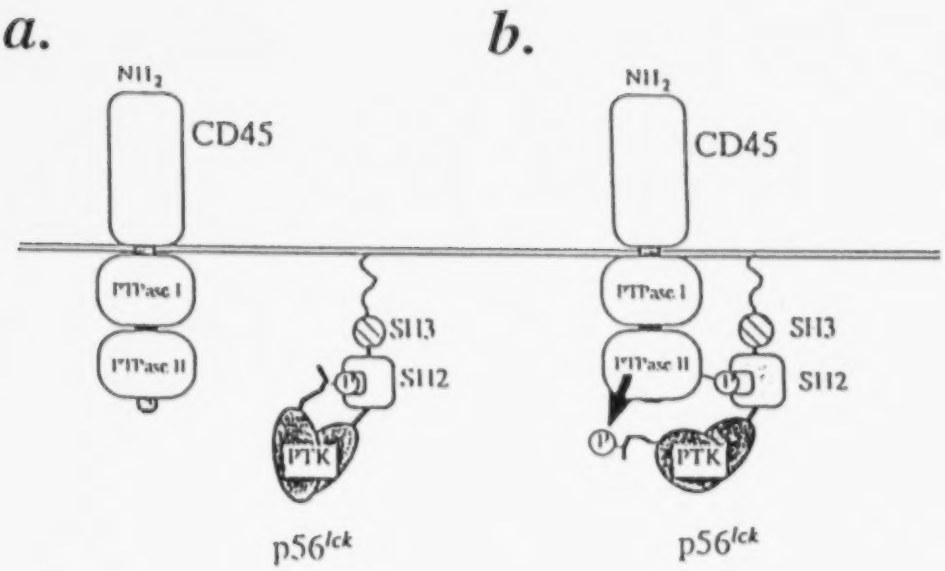

FIGURE 42. Proposed mechanism for the tyrosine phosphorylation -and SH2-dependent interaction between CD45 and Src family PTKs. (a) CD45 is unphosphorylated, and $P56^{kk}$ is phosphorylated at Tyr-505 and is suppressed by intramolecular binding of the phosphorylated Tyr-505 to the SH2 domain. (b) Upon phosphorylation of CD45 at Tyr-1193 (e.g., by $p50^{csk}$), the SH2 domain of $p56^{kk}$ binds this site, liberating the still-phosphorylated Tyr-505, which now is accessible to CD45-mediated dephosphorylation. This Figure is from Ref.24.

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